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TITLE: Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers as Quest for Therapeutic Drug Targets in Rat Model of Blast Overpressure Wave Exposure

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14. ABSTRACT A frequent cause of traumatic eye injuries to soldiers is exposure to blast shock waves; and it can involve cellular damage to the retina as well as brain visual centers. Since there are relatively few animal studies that have studied this, there is an urgent need to advance the characterization of blast induced visual system injuries and identify potential drug therapies. Inflammation plays a key role in the destruction of injured neuronal tissues, as carried out by immune cells; and thus is a promising target. Scope and timing, however, of this process must be better understood. Our study uses an adult rat model of eye and brain injuries, as produced by exposure to simulated blast waves in a shock tube. Rats are kept on an omega-3 polyunsaturated fatty acid deficient diet, which promotes inflammation. Conversely, some are fed an omega-3 enriched diet by ocean fish oil supplementation. Up to one month after blast, eye (retina) and brain damage is assessed by electroretinography (ERG), visual acuity task, magnetic resonance imaging (MRI), histopathology, and immunoassay arrays for inflammation signaling factors. Our findings reveal that blast wave exposure leads to acute (i.e., within 3 days) impairment of visual function with underlying infiltration of activated immune cells (i.e., macrophages and microglia) in the brain and retina, which is accompanied by elevated cytokines and degeneration of neurons. Despite having potent anti-inflammatory properties, high doses of dietary omega-3 fatty acids showed slight if any ability to alleviate these acute injury events. Chronic events, however, maybe more amendable to other functions of omega-3 fatty acids, such as the rebuilding of neuronal cell membranes. Thus, we plan to extend our evaluations over more prolonged post-blast time periods. Overall, our mission is to provide results that will lead to selection and animal testing of new drugs for blast-induced damage sustained to the visual system of US Army personnel.					
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I. INTRODUCTION:

The objective of the project is to identify mitigation and treatment strategies for blast-related traumatic injuries to the neuronal components of the visual system, as frequently suffered by US Military personnel in current fields of operation. These can involve grave damage to the eyes (retina) as well as brain visual processing centers. Despite the difficult long term disability that loss of vision represents, there are relatively few animal studies that have characterized blast-induced neuronal injuries to the visual system, and searched for promising drug based therapies. In rats exposed to blast over pressure waves, we have already documented marked retinal signaling dysfunction caused by photoreceptor degeneration, which extends to central brain pathways associated with vision (e.g., optic tract, lateral geniculate nucleus, and superior colliculus). Based on our own preliminary data and that published by others showing accumulation of activated microglia and macrophages in the retina and brain following blast, we hypothesize that immune cell mediated processes play a primary role dictating the extent of blast-induced neurodegeneration in the visual system. Thus, our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in retina and brains of adult male rats after blast wave exposure, using a compressed air driven shock tube, so as to discern potential drug targets and therapeutic windows. To effectively utilize this model to identify therapies, it is crucial to understand the biochemistry underlying the injuries' progression. In particular, scope and timing of immune cell infiltration and cytokine release requires rigorous characterization. Nutritional impact on blast injury vulnerability also will be studied under pro-inflammatory conditions, i.e., a diet deficient versus one enriched in omega-3 polyunsaturated fatty acids, including both docosahexaenoic and eicosapentaenoic acids (DHA and EPA, respectively), as provide by daily supplementation with high dose ocean fish oil. This will enhance our ability to monitor immune cell processes in neuronal structures for discovery of unique drug targets. We will assess the health status of the rat's retina and brain following the blast insult at acute and chronic time points, i.e., 3, 7, 14, and 28 days. Outcome measures that will be used are electroretinography (ERG) for neuronal signaling to a light stimulus; visual acuity task (optokinetics) for behavioral reflex to object movement; Magnetic Resonance Imaging (MRI) for in situ immune cell tracking and structural anatomy; histopathology for immune activation and neuronal cell degeneration, and immunoassay arrays for cytokine / chemokine levels. Overall, our objective is to provide extensive data that will identify translatable therapies targeting blast-induced vision impairments sustained by Warfighters. Thus, these studies may provide high impact advancements to the field of Military Medicine that can help lessen the burden experienced by thousands of severely visually impaired service members and their families, which can spill over into improving civilian medicine. Treatment and rehabilitation of visual system injuries in veterans has annually cost the US economy billions of dollars, and an enhanced understanding of etiology and effective treatment strategies could help with reducing a significant portion of this financial predicament. Thus, the proposed research has enormous benefit to supporting the mission and goals of the US Military.

- **KEYWORDS:** Rat, blast wave, neurotrauma, neurodegeneration, eye, retina, brain, inflammation, macrophage, microglia, astrocyte, neuron, omega-3 polyunsaturated fatty acid, electroretinography (ERG), visual acuity, optokinetics, magnetic resonance imaging (MRI), histopathology, immunoassay array, and cytokines.
- **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

- **What were the major goals of the project?**

The goals for year two of the project as detailed in the statement of work were as follows:

- 1) Place 24 rats on an omega-3 fatty acid deficient diet for one month, as given alone or with fish oil supplementation administered by daily oral gavage. Projected time period for this task was months 12 - 14.
- 2) Give the rats baseline ERG and visual acuity examinations and then expose half of them to double blast over pressure waves (1 min interval) in a compressed air driven shock tube. The second half will be utilized as shams (i.e., uninjured controls). Projected time period for this task was months 12 - 14.
- 6) Repeat ERG and visual acuity examinations on all rats at 3, 7, and 28 days post-exposure. Projected time period for this task was months 12 -14.
- 7) A subset of 8 rats are euthanized (i.e., at 28 days post-exposure) and perfused with saline and then paraformaldehyde. The fixed carcasses are sent to our outside collaborators at the University of Pittsburgh's Animal Imaging center (Dr. T. Kevin Hitchens' lab) for MRI analysis (immune cell tracking and structural anatomy) of the eyes (retinas) and brains. Projected time period for this task was months 12 - 14.
- 8) The heads from the above rats are returned to us, and the eyes and brains are removed and submitted to the local company FD Neurotechnologies for histopathology processing. Returned slides are assessed under a microscope for immune cell activation and neuronal cell degeneration in the retina and brain visual centers. Projected time period for this task was months 12 - 14.
- 9) A subset of 12 rats are euthanized (i.e., at 28 days post-exposure) and fresh plasma, retinas, and brains are collected. These tissues are then subjected to immunoassay arrays to determine the levels of various cytokines that are present. The projected time period for this task was months 12 - 14.
- 10) Bring in a fresh group of 24 rats and repeat steps 4 - 9, as above, to obtain outcome measures for retina and brain status at baseline and 28 days post-exposure (i.e., ERG, visual acuity, MRI, histopathology, and cytokine / chemokine levels), under omega-3 fatty acid deficient and enriched diets. Projected time period for this set of tasks was months 15 - 19.
- 11) Wrap up all sample and data processing and begin writing of publications for submission to US Army approved scientific journals. Submit quarterly and annual reports as required along the way of the study. Projected time period for this set of tasks was months 19 - 24.

In summary, the three basic milestones for the 2nd year of the project were as follows:

Milestone #1: Data reported for blast wave induced retina and brain inflammation at 28 days post-exposure (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays), by month 15 and finished at 19.

Milestone #2: Data reported for shams, i.e., non-injured controls, at 28 days post-exposure to mock blasting (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays), by month 15 and finished at 19.

Milestone #3: Wrap up the study and initiate publication of the results in appropriate peer reviewed scientific journals, by month 19 and finished at 24.

- **What was accomplished under these goals?**

During the first year of the study, we had fallen 5 -6 months behind schedule due to problems obtaining the animal protocol and initiating the CRADA agreements to do the MRI analysis work on the animals with our collaborators, Dr. T. Kevin Hitchens' group at Carnegie Mellon University. He took a new position at the McGowan Institute's Animal Imaging Center at the University of Pittsburgh; and, thus, we had to resubmit the contract agreements and wait until he had moved into his new laboratory to send samples for analysis. This delay lead to a reset of the SOW schedule to the milestones described under the first year, which were to accomplish gathering data primarily at 3 and 7 days post-blast (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays). Thus, in the second year of the study, we focused on moving these goals forward to completion, and pushing forward our original plans of doing rats at a 28 day post-blast time point along with corresponding shams into a 6 month no cost extension period.

In the 2nd year of the project, our primary accomplishments were:

- 1) Ordered and received the following groups of animals for the project's experiments: 8 rats on 15 September 2015 (end of first year); 8 rats on 01 October 2015; 12 rats on 24 November 2015; 12 rats on 27 January 2016; 18 rats on 10 March 2016; 18 rats on 26 April 2016; 12 rats on 24 May 2016; and 12 rats on 21 July 2016. This constitutes 8 experimental groups, for a grand total of 100 animals utilized.
- 2) After one week of acclimation to our holding facility, placed the animals for at least one month (i.e., 4 - 5 weeks) on a continuous diet deficient or enriched in omega-3 polyunsaturated fatty acids. The division of animals within in each group between the two diets was always an even 50/50 split.
- 3) Provided placebo (soybean) and ocean fish oil supplementation to the rats on long chain omega-3 polyunsaturated fatty acid deficient and enriched diets, respectively, as given once daily by oral gavage, except on Saturdays due to staff availability problems. Thus, the animals were treated 6 days per week.
- 4) Recorded daily body weights of the rats, during the placebo and fish oil treatments. Initial and final weights were obtained for 49 placebo and 50 fish oil treated rats. One

placebo rat spontaneously died early on in the treatment regime.

5) Checked the blood glucose levels of rats once per week (i.e., on Friday or Monday) over one month by tail stick and a hand held diabetic-glucose meter. Only, the first four experimental groups were examined yielding a total of 19 placebo and 20 fish oil treated animals. As a control group, the blood glucose levels of 14 normal rats was randomly tested, which were from our other projects and fed a standard “house” chow diet containing sufficient long chain omega-3 fatty acids.

6) After one month of feeding, exposed rats while under anesthesia once to double blast over pressure waves (1 minute interval; 20 psi) using a compressed air driven shock tube. Blast wave exposures for the 9 experiment groups were done on 21 October 2015, 02 November 2015, 04 & 05 January 2016, 08 March 2016, 18 & 19 April 2016, 06 & 07 June 2016, 11 July 2016, and 29 August 2016. Overall, this produced exposure survivors for 26 placebo blasted, 10 placebo sham, 30 fish oil blasted, and 11 fish oil sham treated rats. Also, following the blasting recorded the rat’s righting reflex, i.e., time to return to full consciousness.

7) Carried out visual acuity exams (i.e., optokinetics) on rats at up to 8 days prior (baseline) to blast exposure and at 2 or 6 days thereafter. Visual acuity data for both right and left eyes was obtained for 10 placebo and 12 fish oil treated rats at 2 days post-blast and 7 placebo and 12 fish oil treated rats at 6 days post-blast. Some shams were also done but not yet analyzed.

8) Carried out electroretinography recordings (i.e., full field flash ERG) on rats at up to 7 days prior (baseline) to blast exposure and at 3 or 7 days thereafter. ERG data for both right and left eyes was obtained for 10 placebo and 12 fish oil treated rats at 3 days post-blast and 7 placebo and 12 fish oil treated rats at 7 days post-blast. Some shams were also done but not yet analyzed.

9) Euthanized rats at ~ 3 or ~ 7 days post-exposure, by exsanguination under anesthesia. Fresh tissues, i.e., eyes (retinas), brains, liver, and plasma were collected for 11 placebo and 13 fish oil treated rats at 3 day post-blast and 6 placebo and 9 fish oil treated rats at 7 days post-blast. Likewise, fresh tissues were taken from 5 placebo and 6 fish oil treated shams. Other rats were perfused with saline followed by paraformaldehyde; and fixed eyes and brains or whole carcasses were collected for 5 placebo and 4 fish oil treated rats at 3 days post-blast and 3 placebo and 3 fish oil treated rats at 7 days post-blast. Likewise, fixed tissues were taken for 5 placebo and 5 fish oil treated shams.

10) Immediately submitted terminal blood samples to the WRAIR Clinical Pathology Department for complete blood cell count (CBC) and chemistry panel work up. Blood work data was obtained for 17 placebo and 17 fish oil treated rats at 3 days post-blast and 8 placebo and 12 fish oil treated rats at 7 days post-blast. Some shams were also done but not yet analyzed.

11) Performed fatty acid composition analysis of liver samples collected from 5 placebo and 5 fish oil treated rats at 7 days post-blast, using lipid extraction and GC/MS methods.

Analysis of samples was completed on 22 February 2016.

12) Performed cytokine concentration determinations, using multiplex immunoassay arrays, on collected fresh brains (mid-brain and cerebellum regions) and plasma. Assays were carried out on 04 December 2015 (5 brains), 29 December 2015 (4 brains), 17 June 2016 (16 brains), 05 August 2016 (19 brains), and 16 September 2016 (36 plasmas). Cytokine data was determined for 11 placebo and 10 fish oil treated rats at 3 days post-blast and 4 placebo and 4 fish oil treated rats at 7 days post-blast. Likewise, shams were done for 9 placebo and 6 fish oil treated animals, which were combined as a single control group. Of the placebo shams, 4 animals came from tissues collected under another project of ours having a similar design.

13) Performed histopathology (H&E and silver stains) and immunohistochemistry (GFAP, Iba-1, and CD68) assessments on collected formaldehyde fixed eyes (retina) and brains. Samples were submitted to FD Neurotechnologies, Inc. (Dr. Fu Du's lab) for processing into slides on 09 November, 2015 (2 rats), 17 May, 2016 (6 rats), and 12 September 2016 (1 rat). Slides were returned to us within two months and assessed under a microscope at our leisure, as by taking representative pictures of cellular aberrations in retinas and brain optic tracts. Histopathology data was obtained for 1 placebo treated rat at 3 days post-blast and 3 placebo and 3 fish oil treated rats at 7 days post-blast. Likewise, shams were done for 1 placebo and 1 fish oil treated rat.

14) Performed magnetic resonance imaging (MRI) on collected formaldehyde fixed whole carcasses. At one day prior to sacrificing the animals, they were intravenously infused with a ¹⁹F-labeled contrast agent that is specifically taken up by activated macrophages. Samples were submitted to the McGowan Institute's small animal imaging center at the University of Pittsburgh (Dr. T. Kevin Hitchens' lab) on 22 June 2016 (8 rats) and 06 September 2016 (8 rats) for ¹H structural anatomy and ¹⁹F immune cell tracking scans of the in situ eyes and brains. Image files were returned to us within one month and assessed for tissue injury sites and macrophage deposits via teleconferences with Dr. Hitchens. MRI data was obtained for 4 placebo and 4 fish oil treated rats at 3 days post-blast. Likewise, shams were done for 4 placebo and 4 fish oil treated rats.

Table 1. Tallies to date of animals fully reported for treatments and outcome measures

Treatments	Visual acuity	ERG	Blood work	Brain / plasma cytokines	Liver / brain fatty acids	Retina histo.	Brain histo.	MRI
Placebo; sham	0	0	0	9 / 9	0	1	1	4
Fish oil; sham	0	0	0	6 / 6	0	1	1	4
Placebo; 3 days post-blast	10	10	17	11 / 11	0	1	1	0
Fish oil; 3 days post-blast	12	12	17	10 / 11	0	0	0	0
Placebo; 7 day post-blast	7	7	8	4	5 / 0	3	3	4
Fish oil; 7 days post-blast	12	12	12	4	5 / 0	3	3	4

Table 1: Outcome measures for retina / brain histopathology and MRI have none or statistically irrelevant numbers of animals completed so far for each treatment. Total input of rats toward shams or double blast exposure for the placebo and fish oil groups was ~ 50 for each diet. This count encompasses all rats utilized in the study so far including any whose data was not included for certain outcome measures. Prior to and/or during blasting, all of these animals were used for reporting extra outcomes of body weight gains, survival rates, righting reflex, and blood work.

Detailed Experimental Methods, Results, and Conclusions:

I. Animals and Dietary Manipulations (Omega-3 Deficient and Enriched Diets):

Materials and Methods:

Adult male Sprague-Dawley rats are obtained from Charles River Labs (Portage, MI). This strain compared to others shows better long term recoveries from neuronal injuries. Initial body weights are ~ 100 g (30 days old) to help limit their final body sizes at our experimental end points. Rats are housed in our animal facility, under tightly regulated environmental conditions. Dietary requirements are provided by custom made chows as detailed below. One month prior to double blast wave injuries and continued thereafter until the experiment's end, rats are fed ad libitum a base diet that is deficient in long chain omega-3 polyunsaturated fatty acids. The diet is a commercial rodent chow that is essentially devoid of all long chain omega-3s including docosahexaenoic acid (DHA) (TestDiet®; "Typical American diet" - #: 5TLN; Purina Mills, LLC). The major goal of feeding the rats an omega-3 deficient diet is to induce a pro-inflammatory state, to aid in seeing changes in our outcome measures post-blast. In accord with this, the "American" diet contains very high amounts of linoleic acid (LA; 8% of calories) an omega-6 fatty acid that is the precursor to inflammation stimulating arachidonic acid and its prostaglandin / leukotriene metabolites. We have shown that feeding this diet to the rats for one month depletes their liver stores of DHA by 65%; however, due to fatty acid conservation mechanisms, the brain DHA remains unperturbed. During the feeding regime, the rats are scale weighed daily to monitor body weight gain. Also, their glucose levels are examined once per week (i.e., Fridays) by a handheld blood glucose monitor (Contour®, Bayer), using a small drop of blood that is drawn via needle prick of the tail.

Rats receive dietary omega-3 fatty acid supplementation using ultra-pure grade fish oil (ProOmega®; Nordic Naturals, Inc). The fish oil contains DHA at 23% by weight; and is given to the rats once daily by oral gavage at a DHA dose of 200 mg/kg/d. This amount of DHA is 4 times the USA-FDA recommendation for normal humans and 2 times that suggested by physicians during recovery from traumatic brain injuries. The fish oil also provides 273 mg/kg/d of eicosapentaenoic acid (EPA; an omega-3); which is converted to DHA and similar anti-inflammatory metabolites. Rats kept on the omega-3 deficient diet receive a placebo preparation (Nordic Naturals, Inc), consisting of soybean oil plus all additives found in the fish oil (i.e., flavors and preservatives). To prepare the fish oil for oral administration, an aliquot appropriate for DHA dosing the rat's body weight (~ 1 ml/kg) is combined with at least 0.5 ml of room temperature instant non-fat milk (10% wt/vol; Carnation - Nestlé). Placebo oil is given using identical volumes. The oil-milk mixture is rapidly agitated and then given to the rat by gavage directly into the stomach, using a 1 cc

syringe fitted with a disposable plastic feeding tube (Harvard Apparatus). The procedure is done by finger collaring the rat, sliding the feeding tube down the throat / esophagus, and quickly depressing the syringe plunger. This method causes minimal discomfort and no harm to the animal.

Results and Conclusions:

We have fully raised a total of 50 rats each for one month on an omega-3 fatty acid deficient or enriched diet (i.e., placebo and fish oil treated, respectively). During the feeding regime we carefully monitored their body weights each day. Shown in Figure 1 is a bar graph of the starting and final body weights for the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 49 and 50, respectively). We found that there were no significant differences or trends detected between the two dietary groups in body weight gain over a one month feeding period, with an average mass increase of 9 g/d or 2%/d. Weight gain in rats is apparently a very analogous measure, with dietary omega-3 fatty acids playing no influence. This is an interesting finding, since omega-3 fatty acids are known to upregulate genes, via PPAR transcription factors, that are involved in the energy metabolism of fat stores. It is doubtful that increasing these already very large group sizes any further will reveal this finding by other investigators. Also shown in Figure 2 is a bar graph of the weekly blood glucose levels for the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 20 each). There were no significant differences in glucose levels detected between the two diets at any week during feeding over one month. As reported before, both dietary groups stayed well within the normal glucose range for rats, which we rigorously found from newly added “house” chow fed rats to be 105 ± 13 mg/dL (dotted line; n = 14). These results satisfactorily prove that our base diet (high fat and carbohydrate with no omega-3s) does not generate insulin imbalance problems in the rats, i.e., pre-diabetic state, which might exacerbate neuronal injuries. Thus, we plan to discontinue the weekly monitoring of their blood glucose levels; which, in turn, will spare the tail veins from damage that might impede the intravenous administration of contrast agent during any future MRI experiments.

Figure 1: Body weight gains of rats over one month on dietary treatments.

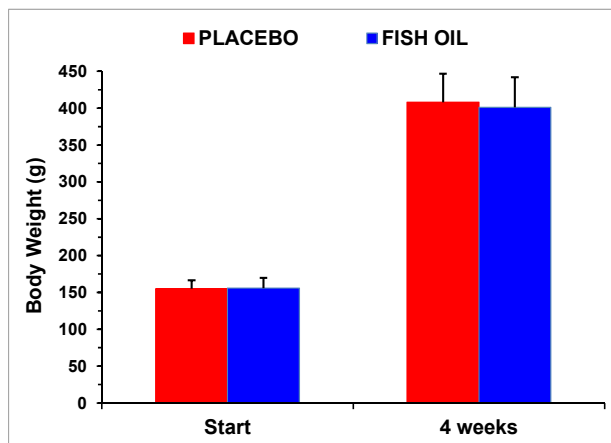


Figure 1. Bar graph for the starting and final body weights of the placebo versus fish oil treated

rats (placebo = red and fish oil = blue; n = 49 and 50, respectively). There were no significant differences detected between the two dietary groups in body weight gain over a one month feeding period, with an average body mass increase of 9 g/d or a 2%/d.

Figure 2: Blood glucose levels of rats over one month on dietary treatments.

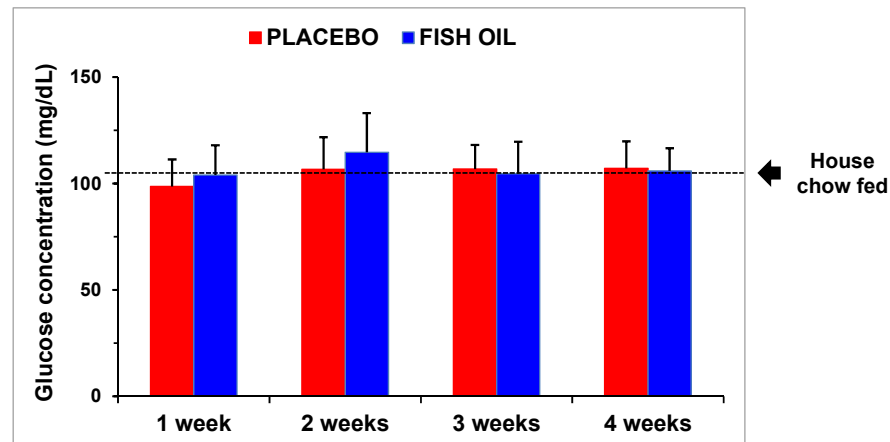


Figure 2. Bar graph for the weekly blood glucose levels of the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 20 each). Dotted line shows average glucose levels of normal “house” chow fed rats (105 ± 13 mg/dL; n = 14). There were no significant differences between any of these three dietary treatment groups.

II. Induction of Eye and Brain Injuries using Exposure to Double Blast Waves.

Materials and Methods:

After at least one month of dietary treatments (i.e., omega-3 deficient and enriched diets; placebo and fish oil, respectively), rats are placed under brief anesthesia using isoflurane gas. Anesthetized animals are placed in a prone transverse position inside a nylon mesh sling that is firmly secured to a metal frame sled. Rats are positioned with right side of body perpendicular to the sled, with left side toward, and hence right eye facing the oncoming blast wave during exposure. In this manner, the left eye serves as a control, expected to incur less severe injuries or none. The rat-loaded sled is inserted down the barrel of a compressed air driven shock tube to a preset position in its forward expansion chamber. The unawake animal is then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 1 min interval), which has been previously shown by us to produce significant neuronal injuries to the retina and brain visual centers. The double blast waves are generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness, to deliver 20 psi of air to the rat’s position, as clamped between the rear compression and forward expansion chambers. Blast waves travel by the rat with a Mach 1.34 shock front, 62 μ sec rise time, 8 msec duration, 126 m/s wind speed, and an acceleration g-force of > 1000 g. Double blasted rats are immediately removed from the shock tube and monitored during recovery. Animals exhibiting stable respiration are returned to their housing cages and laid on their sides. Time for the animal to regain an upright prone posture (i.e., righting reflex)

is recorded, as an indication of its return to consciousness. Blasted rats are subjected at 3, 7, 14, and 28 days post-injury to blood work, visual acuity, ERG, fatty acid composition, and histopathology outcome measures, as to be described below.

Results and Conclusions:

We have exposed a total of 39 placebo and 39 fish oil treated rats to double blast over pressure waves. Shown in Figure 3 is a table of the long term survival numbers and a bar graph of the righting reflex (i.e., time to regain an upright posture) for the placebo versus fish oil treated rats, following double blast exposure (placebo = red and fish oil = blue, n = 39 and 39 and 26 and 29, respectively). Similar to our previous report, we found the incidence of blasted related deaths (immediate and delayed combined) for the placebo versus fish oil treated rats was 33 and 23%, respectively; suggesting omega-3 fatty acid deficiency slightly lowers the animal's resiliency to blast. We did chi square analysis on the survival numbers and found, however, they are still not significantly different at this point. Likewise, while no significant differences were detected between the two diets for righting reflex, there is a steadfast trend for the placebo group to have a slower return to consciousness, which also implies they are not as blast resilient. Indeed, for both groups, animals that displayed a delayed death (> 3 hours) from blast-injury complications typically took a prolonged time to regain consciousness (n = 3, each).

Figure 3: Survival rates and Righting Reflex of rats following double blast exposure.

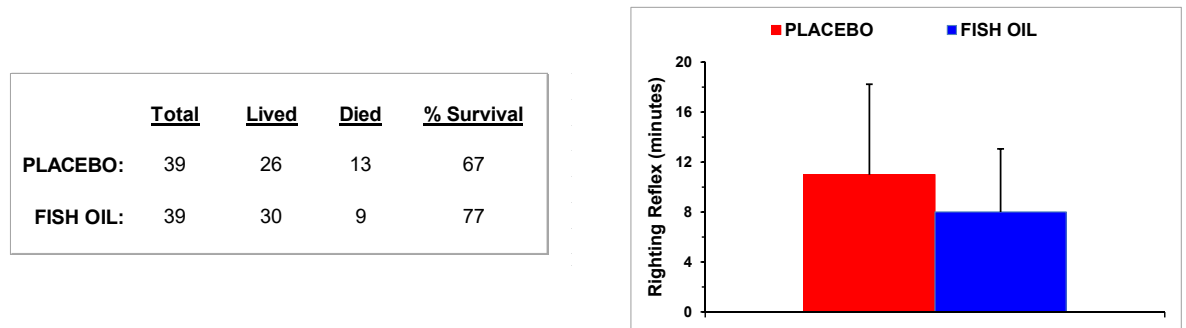


Figure 3. Bar graph and table for the survival rates and righting reflex of the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 39 and 39 and 26 and 29, respectively) shortly following double blast exposure. There were no significant differences between dietary treatment groups, as by chi square and t-test, respectively.

III. Visual Acuity (Optokinetics) Assessments of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

To judge their visual discrimination behavior capacities rats undergo visual acuity examinations using an optokinetics device (Optomotry unit; Cerebral Mechanics Inc). Measurements are taken at baseline at least two days prior to double exposure and at 3, 7, 14, and 28 days thereafter. To do the test the conscious rat is placed unrestrained on a

12 cm diameter pedestal raised 20 cm above the floor inside a 45 x 45 x 45 cm chamber, where it is surrounded on all four sides by LCD monitors that project a virtual rotating vertical black and white bar pattern having an adjustable spatial frequency (i.e., sine wave grating in cycles/degree). Flickering movement of the bar pattern immediately induces an eye tracking reflex (nystagmus) in the animals, which is viewed by an overhead mounted video tracking camera connected to an external computer with monitor. According to the manufacturer, a cursor is placed on the rat's forehead to keep the rotation of the cylinder centered according to the animal's perception, thereby "clamping" the effective spatial frequency of the grating. Visual acuities can be obtained in minutes in animals with no previous exposure to the task, and measurements can be repeated regularly even within the same day. System calibration, video capture, and an array of psychophysical testing methodologies (e.g., acuity and contrast thresholds) are managed by the instrument's controlling software.

For assessment of visual acuities, rotation of the bar pattern is set to a fixed spatial frequency, at which normal rats can readily see (e.g., 0.04 cycles/degree at 100% contrast) and the rotation speed is increased stepwise to narrow the apparent width of the bars. Contribution of each eye is resolved by driving the pattern's rotation in opposite directions, i.e., clockwise and counter clockwise for left and right eyes, respectively. Eye pursuits are judged by watching for reflexive movements (side flicks) of the entire head, which are aligned with the direction of the stimulus rotation. Visual acuity thresholds are manually found by steadily increasing over 15 min the spatial frequency of the bar pattern, until the animal no longer exhibits head tracking movements. The instrument helps the user reliably lock on the acuity thresholds by keeping the current spatial frequency hidden and using up and down speed reiterations in line with "no" and "yes" responses for head tracking movements via the computer's key board. The test ends when the user inputs consistent responses of "no" for head tracking, and the acuity threshold is revealed. In this manner, the acuities are measured with an accuracy of 0.001 cycles/degree.

Results and Conclusions:

We have completed base line and post-injury visual acuity assessments for a total of 17 placebo and 24 fish oil treated rats at 2 or 6 days out. We had to shift the original planned times (i.e., 3 and 7 days) back one day, due to inability to do the visual acuity and ERG testing on the same day. Shown in Figure 4 are the bar graphs of the right and left eye visual acuities for placebo versus fish oil treated rats, at 2 and 6 days (top and bottom panels) following double blast exposure (placebo = red and fish oil = blue, n = 10 and 12 and 7 and 12, respectively). For the right and left eyes, we have found for fish oil treated rats a significant decline in visual acuities from baseline (13%, each) at 2 days post-blast. There is also an apparent trend, at 2 and 6 days post-blast for the right eye of placebo treated rats to have lower acuities than their baseline and from those given fish oil. The right eye deficits are consistent with the blast being directed towards that side of the animal. This again suggests that visual acuity losses are a chronic event following blast wave injury; and deficits will become more robust as we examine points further out, i.e., 14 - 28 days. It is known that losses in visual acuity are compensated for by plasticity of the brain and retina neural networks and thus tend to gradually manifest over time.

Figure 4: Visual acuity values of double blasted rats at 2 and 6 days post-exposure.

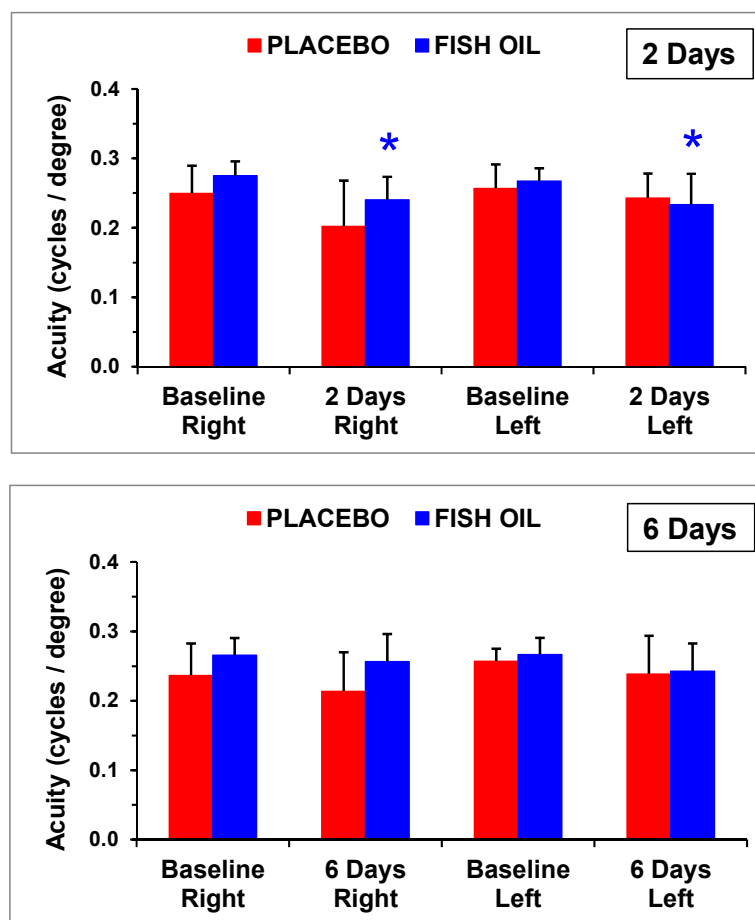


Figure 4. Bar graphs for the right and left eye visual acuities of placebo versus fish oil treated rats at their respective baselines and 2 and 6 days (top and bottom panels) following double blast (placebo = red and fish oil = blue; n = 10 and 12 and 7 and 12, respectively). At 6 days out, for the right and left eyes there were no significant differences between dietary groups or from their baselines. *p < 0.05, significant difference from baseline, as by t-test.

IV. Electroretinography (ERG) Recordings of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

To determine the retina signaling response status of their eyes, at least 1 day prior to (baseline) and 3, 7, 14 and 28 days after double blasting, rats will undergo electroretinography (ERG) examinations, i.e., full field flash testing. This will be done using an ERG machine (Color Dome Full Field Ganzfeld unit; Diagnosys LLC). Full field flash ERG (standard ERG) detects the light stimulus evoked potentials arising from retinal photoreceptors and their synaptic joined bipolar and amacrine neurons. An ERG recording consists of two distinct waveform components, i.e., the initial a-wave (negative deflection) representing hyper-polarization of the photoreceptor cells, which results from a closure of their ion channels and turn off of neurotransmitter release, and a following b-wave (positive

deflection and slow decay) arising from an opposite depolarization of the bipolar and amacrine neurons.

To prepare the rats for the ERG exams, they are first dark adapted overnight (16 hours) in a sealed darkroom to prime their retinal responses to light (scotopic ERG). We have found, however, that more robust and consistent responses are obtained, if possible, with an overnight adaptation (16 hours). All subsequent ERG procedures are carried out in the darkroom under red photography lights to which rat retinas don't respond; since they lack photoreceptors for red color vision. Each rat is placed under continuous isoflurane anesthesia, as delivered via a nose cone. The rat's pupils are then dilated using saline drops containing 0.5% tropicamide and 2.5% phenylephrine. As a corneal anesthetic, each eye receives 0.5% propracaine drops. The anesthetized animal is then laid prone on the exam table of the ERG machine. A ground electrode is fixed to the base of the tail, reference to each cheek, and recording to each eye's cornea. Our ERG machines use sub-dermally inserted pin electrodes (12 mm) for the ground and reference wires. The recording electrodes, which are fine gold-wire loops on movable arms, are situated directly onto the corneas using drops of 2% methylcellulose solution as a conductor and cushion. A light stimulus dome (LED flash lamp) is then lowered over the upper body of the animals. The rat is then presented with a series of white light flashes of increasing intensity at 0.1, 1, 3, and 10 cd.s/m² illuminations, 5 msec durations, and 30 - 60 sec intervals. The flashes are done in triplicate (2 sec intervals). These parameters are ideal for response detection of retinal photoreceptors involved in black and white vision. Results are reported as peak amplitudes of the a- and b-waves at the 10 cd.s/m² flash, which is a direct indication of retinal neuron health.

Results and Conclusions:

We have completed base line and post-injury ERG exams for a total of 17 placebo and 24 fish oil treated rats at 3 or 7 days out. Shown in Figure 5 are the bar graphs of the right and left eye a-wave amplitudes (i.e., photoreceptor response at a 10 cd.s/m² flash stimulus) for the placebo versus fish oil treated rats, at 3 and 7 days (top and bottom panels) following double blast exposure (placebo = red and fish oil = blue, n = 10 and 12 and 7 and 12, respectively). The results show that there is a significant decrease in a-wave amplitudes compared to baseline values for the right and/or left eyes of the placebo and fish oil treated rats at 3 days out (39, 45, and 51%, respectively). In contrast, at 7 days post-blast just the right eyes of the placebo group had a significant decline from baseline (32%), along with a trend for their left eyes to be impaired. In this case, the two dietary groups possess more similar baseline values. Overall, our findings again show that blast exposure is damaging the retina neurons in both eyes; but now, it is less convincing omega-3 fatty acids are effective at alleviating this at the acute stage. The starting values of the fish oil group for both eyes, however, have a trend to be slightly higher than the placebo groups, which is in line with omega-3 fatty acids promoting general retinal health.

Figure 5: ERG amplitudes (a-wave) of double blasted rats at 3 and 7 days post-exposure.

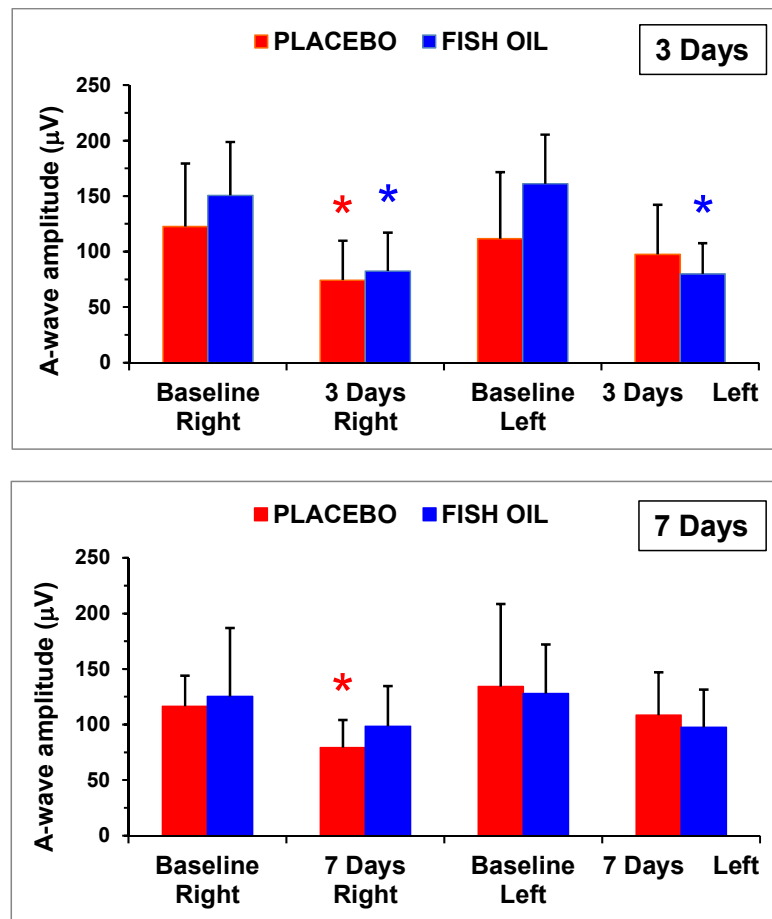


Figure 5. Bar graph for the right and left eye a-wave amplitudes (10 cd.s/m² flash stimulus) of placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 10 and 12 and 7 and 12, respectively) at baseline and 3 and 7 days following double blast. *p < 0.05, significant difference from baseline, as by t-test.

V. Blood Work and Fatty Acid Assessments of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Rats from the placebo and fish oil dietary treatment groups are euthanized at 3, 7, 14, and 28 days post-blast exposure for collection of fresh tissues, i.e., plasma, brain, and retinas. Animals will be deeply anesthetized by isoflurane inhalation and then subjected to terminal blood exsanguination, using cardiac puncture as done with a syringe through the chest. Excess blood is collected in EDTA and heparinized vacuum tubes kept on ice. Some whole blood is submitted to the WRAIR Department of Clinical Pathology for complete blood-cell count (CBC; hemocrit, platelets, and white blood cell types) and chemistry panel (cholesterol, triglycerides, alanine aminotransferase, and aspartate aminotransferase). The biomarkers selected for the chemistry panel are mainly a measure of liver function, in light of the high fat / low omega-3 base diet we are giving the rats. Remaining blood is centrifuged to obtain plasma. Once respiration and heartbeat have ceased, the rats are

subjected to guillotine decapitation. Whole brains are removed from the heads, washed with saline, dissected into cerebellum, cortex, and midbrain regions, and then quick frozen on dry ice. Eyes are enucleated and dissected to obtain the retinas. An eye cup is formed by cutting off the eye's anterior segment (i.e., cornea, iris, and lens) and the retina is isolated using jeweler's forceps and washed with saline. Retinas from both eyes are combined in one tube, and quick frozen on dry ice. A section of liver lobe, for fatty acid profile purposes, is also removed and quick frozen. All tissue samples are stored frozen at -80oC for future analysis.

For fatty acid analysis, liver samples are homogenized, by probe sonicator, into methanol containing butylated hydroxytoluene (BHT) as an antioxidant. Prior to homogenization, heptadecanoic acid (17:0) is added as internal standard for later quantification purposes. Total lipids are extracted from the tissue homogenates using chloroform, as partitioned out by addition of KCl solution. Chloroform phases containing crude total lipids are converted to fatty acid methyl esters (FAMES) by reaction with BF₃ in methanol. FAMES are extracted into hexane and then analyzed on an Agilent Technologies 5975C / 7890A gas chromatograph - mass spectrometer (GC/MS) system. Mass detector derived peaks (selective range, total ion count) are identified by retention time comparison with a standard mixture of FAMES, which includes all omega-3 and omega-6 polyunsaturated fatty acids of interest. These are also used to derive ionization efficiency response factors for each fatty acid. Concentrations of fatty acids in the original tissue sample (g per wet wt.) are determined by direct proportional comparison of their peak areas to that of the added 17:0 internal standard.

Results and Conclusions:

We have done the blood work, i.e., complete blood count (CBC) and chemistry panel, for a total of 25 placebo and 29 fish oil treated rats at 3 or 7 days out. Shown in Figure 6 is a table for the CBC and chemistry values of terminal blood for the placebo and fish oil treated rats at 3 and 7 days following double blast (n = 17 and 17 and 8 and 12, respectively). Listed at the right side are the various hematological factors, along with normal ranges, which were assessed, e.g., white blood cells (WBC), hemocrit (HCT), and triglycerides (TG). Those factors whose average is substantially above normal are highlighted light blue; and corresponding amount of abnormal rats are shown inside red brackets. Functionally, most crucial to our study are the monocytes (MONO) levels, which can transform into neuron destroying macrophages during rampant inflammation. Monocyte levels in both dietary treatment groups were found to be elevated well beyond normal range at 3 and 7 days post-blast. While the two dietary treatment groups did not significantly differ in monocyte levels, the placebo treated trended to have higher counts at both days post-blast, as well as for total white blood cells, neutrophils, lymphocytes, and basophils at some of these days. As previously found, triglyceride levels were abnormally high at 3 and 7 days post-blast in both diet groups; but they were significantly lower in the fish oil group at 3 days out. Likewise, cholesterol levels were abnormally high in the two dietary treatment groups only at 3 days post-blast, with significantly lower values in those given fish oil. Interestingly, AST and ALT enzyme levels were not abnormal under all treatment conditions, indicating liver function is not drastically impaired; however, the fish oil group trended to have lower AST levels at both days out. To confirm this, we also

looked at the AST to ALT ratios; which are often considered to be a better gauge of liver health, since ALT can be highly expressed in other tissues. We found that the AST to ALT ratios were slightly abnormal in all but the fish oil group at 3 days out, signaling that some underlying liver problems may be present. Overall, the results of the blood work fall in favor with fish oil as being a blast-injury therapeutic, but this would be as only measured against systemic inflammation and metabolic imbalances, and not directly towards neuronal disturbances within the retina or brain.

We have also determined the liver fatty acid compositions from 5 placebo and 5 fish oil treated rats taken at 7 days post-blast. Shown in Figure 7 is a table for the liver fatty acid composition values of placebo versus fish oil treated rats, at 7 days following double blast exposure (n = 5 and 5, respectively). Content of each fatty acid is expressed as a percentage of the total. The most crucial of these to our study are the major biological omega-6 and omega-3 fatty acids: arachidonic acid (ARA; 20:4 ω -6), eicosapentaenoic acid (EPA; 20:5 ω -3), and docosahexaenoic acid (DHA; 22:6 ω -3). We found that in the livers of the fish oil versus placebo treated rats the levels of DHA and EPA were significantly increased (5 and 20-fold, respectively). This was reflected in a significant decrease of the liver's omega-6 to omega-3 and ARA to DHA ratios (5 and 7-fold, respectively). Overall, this supports that providing fish oil to the rats for one month dramatically boosts the omega-3 fatty acid index of their tissues, which is known to greatly suppress inflammation processes. The abundant DHA in the fish oil treated animal livers would also be available for release to the brain to help build new neuronal membranes. Thus, our fish oil treated rats should be more resilient to blast induced neuronal injuries. Interestingly, the levels of ARA in the liver were nearly identical between the two dietary treatment groups. Production of this omega-6 fatty acid should have been greatly diminished by the high omega-3s levels. ARA is also the direct precursor for generation of inflammation stimulating prostaglandins and leukotrienes. Thus, it appears that the one month feeding period of fish oil that we used may not be enough to reap the full anti-inflammatory benefits of the increased tissue omega-3 fatty acid levels. We have not looked, however, at the brain (e.g. occipital cortex) and retina levels of ARA, EPA, and DHA to see if they are moving in an even more favorable direction towards halting neuroinflammation.

Figure 6: Blood work of double blasted rats at 3 and 7 days post-exposure.

	3 DAYS POST		3 DAYS POST		7 DAYS POST		7 DAYS POST		Normal Ranges:	
	PLACEBO (n = 17)		FISH OIL (n = 17)		PLACEBO (n = 8)		FISH OIL (n = 12)			
Blood Test	Ave	SD	Ave	SD	Ave	SD	Ave	SD		
WBC (per μ l):	7754	\pm 2494	6499	\pm 2789	7179	\pm 2006	6540	\pm 2826	WBC	3300 - 8700
NEUT (per μ l):	884	\pm 628	839	\pm 608	778	\pm 415	683	\pm 367	NEUT	300 - 1700
LYMPH (per μ l):	6341	\pm 2197	5221	\pm 2266	5813	\pm 1984	5385	\pm 2202	LYMPH	2600 - 7100
MONO (per μ l):	456	\pm 189 (16)	361	\pm 205 (11)	490	\pm 232 (7)	378	\pm 264 (9)	MONO	0 - 200
EO (per μ l):	68	\pm 23	81	\pm 48	97	\pm 79	93	\pm 49	EO	0 - 100
BASO (per μ l):	4	\pm 5	3	\pm 5	3	\pm 5	2	\pm 4	BASO	0 - 20
HCT (% vol):	41	\pm 3	41	\pm 2	39	\pm 2	41	\pm 2	HCT	33 - 45
PLT (per ml):	976	\pm 120	1008	\pm 143	1011	\pm 97	1004	\pm 146	PLT	493 - 1124
CHOL (mg/dL):	96	\pm 25 (7)	# 74	\pm 17 (2)	79	\pm 13	68	\pm 14	CHOL	20 - 92
TG (mg/dL):	158	\pm 65 (12)	# 112	\pm 51 (10)	196	\pm 102 (6)	193	\pm 62 (11)	TG	27 - 108
AST (U/L):	101	\pm 39	95	\pm 38	82	\pm 15	73	\pm 11	AST	39 - 110
ALT (U/L):	43	\pm 10	49	\pm 17	37	\pm 6	36	\pm 5	ALT	20 - 61
AST / ALT:	2.4	\pm 0.6 (11)	2.0	\pm 0.6	2.3	\pm 0.3 (6)	2.1	\pm 0.3 (5)	AST / ALT	1.0 - 2.0

 = above normal range
 Red brackets are the amount of rats above normal
 # p < 0.05; placebo vs. fish oil, as by t-test

Figure 6. Table for CBC and chemistry of blood from placebo versus fish oil treated rats at 3 and 7 days following double blast (n =17 and 17 and 8 and 12, respectively). Listed at the right side are the hematological factors examined, along with their normal ranges, i.e., white blood cells (WBC), hemocrit (HCT), platelets (PLT), cholesterol (CHOL), triglycerides (TG), aspartate amino transferase (AST), alanine aminotransferase (AST), and ratio of AST to ALT (AST / AST). White blood cells are broken down into sub-types of neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), and basophils (BASO). Those whose average is substantially above normal range are highlighted in light blue; and amount of abnormal rats are shown inside red brackets. # p > 0.05 (highlighted yellow); significant difference between dietary treatment groups, as by t-test.

Figure 7: Liver fatty acid composition of double blasted rats at 7 days post-exposure.

Fatty Acids	PLACEBO		FISH OIL		
	Ave	SD	Ave	SD	
C14:0	1.3	± 0.4	1.1	± 0.3	
C16:0	21.2	± 1.0	20.8	± 1.4	
C16:1 ω -9	3.3	± 1.4	2.4	± 0.7	
C18:0	7.9	± 2.0	8.8	± 2.2	
C18:1 ω -9	26.5	± 1.5	23.3	± 1.9	# ↓
C18:1 ω -7	4.1	± 0.8	3.4	± 0.5	
C18:2 ω -6	24.3	± 4.3	23.0	± 1.0	
C18:3 ω -6	0.2	± 0.05	0.14	± 0.02	# ↓
C18:3 ω -3	1.2	± 0.45	1.2	± 0.2	
C20:0	0.05	± 0.04	0.04	± 0.06	
C20:1 ω -9	0.4	± 0.2	0.4	± 0.1	
C20:3 ω -6	0.5	± 0.1	0.5	± 0.1	
C20:4 ω -6	7.6	± 2.5	6.0	± 1.5	
C20:5 ω -3	0.10	± 0.03	2.0	± 0.4	# ↑
C22:0	0.04	± 0.03	0.04	± 0.04	
C22:4 ω -6	0.13	± 0.08	0.06	± 0.01	
C22:5 ω -6	0.03	± 0.01	0.03	± 0.04	
C22:5 ω -3	0.13	± 0.06	1.8	± 0.7	# ↑
C24:0	0.04	± 0.02	0.05	± 0.03	
C22:6 ω -3	0.9	± 0.3	5.0	± 1.0	# ↑
C24:1 ω -9	0.05	± 0.03	0.04	± 0.05	
% DHA of total FA:	0.9	± 0.3	5.0	± 1.0	# ↑
DHA conc; mg/g:	0.7	± 0.2	3.2	± 1.1	# ↑
ω -6 / ω -3 ratio:	14.9	± 2.9	3.1	± 0.6	# ↓
ARA / DHA ratio:	8.7	± 2.6	1.2	± 0.5	# ↓
** DHA concentrations are expressed as mg per g wet wt. of tissue.					
# p ≤ 0.05, for placebo vs. fish oil group, as by t-test.					
<div style="display: flex; align-items: center;"> <div style="width: 20px; height: 10px; background-color: #90EE90; margin-right: 5px;"></div> = Omega-6 polyunsaturated fatty acid </div> <div style="display: flex; align-items: center;"> <div style="width: 20px; height: 10px; background-color: #FFB6C1; margin-right: 5px;"></div> = Omega-3 polyunsaturated fatty acid </div>					

Figure 7. Table for the liver fatty acid composition values of placebo versus fish oil treated rats, at 7 days following double blast exposure (n = 5 and 5, respectively). The individual omega-3 and omega-6 fatty acids (highlighted pink and light green, respectively) detected were C18:2 ω -6 (LA), C18:3 ω -6 (GLA), C18:3 ω -3 (ALA), C20:3 ω -6 (DHGLA), C20:4 ω -6 (ARA), C20:5 ω -3 (EPA), C22:4 ω -6 (DTA), C22:5 ω -6 (DPA), C22:5 ω -3 (DPA), and C22:6 ω -3 (DHA). The most crucial fatty acids for our study are arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). General content of each fatty acid is expressed as a percentage of the total. Absolute concentration of DHA is expressed as mg per g wet weight of tissue. # p > 0.05; significant difference between dietary treatment groups, as by t-test. Blue shaded arrows indicate direction of change.

VI. Cytokine Assessments of Brain and Plasma from Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

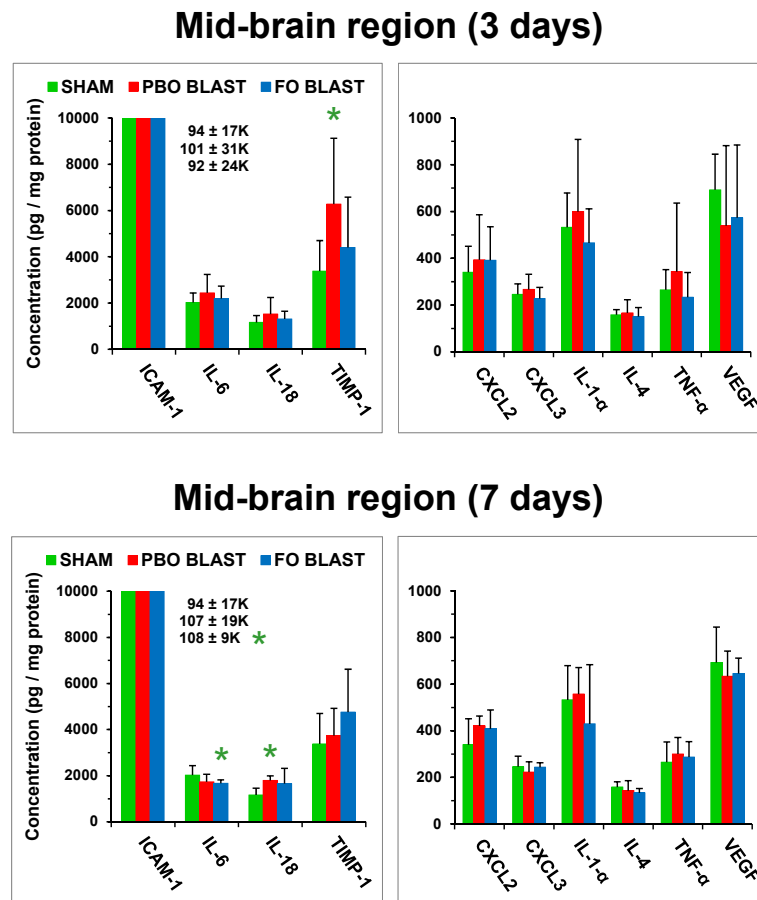
Frozen brain regions are partially thawed and then uniformly representative sections of cerebellum and mid brain are cut out with a scalpel blade. Total protein extracts (lysates) are made for the brain regions, by homogenization into a cell lysis buffer (T-PER; Thermo Fisher Scientific), followed by centrifugation to obtain the supernatant containing the cytokines. Frozen plasma is thawed and analyzed without additional processing. Samples are screened, using kits for immunoassay arrays (R&D Systems, Inc), for simultaneous levels of 11 distinct cytokines, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-10, IL-18, TIMP-1, TNF- α , and VEGF. These factors were chosen by us as a customized array for those that are already known to be upregulated during neuroinflammation. The array consists of a 96 well micro-titer plate containing color-coded polystyrene beads coated with analyte-specific capture antibodies, as well as biotin-streptavidin-PE conjugate detection antibodies. A dual-laser flow-based detector (Luminex technology) is used to precisely quantify the doubly tagged cytokines alongside standard curves made for each. Brain results are reported in pico-grams per milligrams of lysate total protein as determined by a colorimetric assay kit (Bio-Rad Corp) or for plasma given as in pico-grams per ml.

Results and Conclusions:

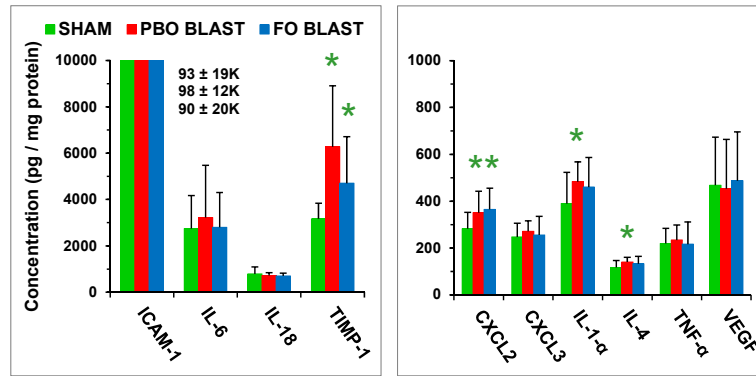
We have done cytokine assays for brains from a total of 11 placebo and 10 fish oil treated rats at 3 days out and 4 placebo and 4 fish oil treated rats at 7 days out. We also determined brain cytokine levels for 9 placebo and 6 fish oil treated shams. Likewise, corresponding plasma was analyzed for all rats done at 3 days out. Shown in Figure 8 are the bar graphs for the brain (i.e., two regions) and plasma cytokine levels of the placebo versus fish oil treated rats at 3 and 7 days following double blast exposure, as well as those for sham controls, (green = shams, PBO; placebo = red, and FO; fish oil = blue; n = 15, 11, and 10 and 15, 4, and 4, as by day, respectively). We analyzed the cytokines found in the mid-brain, which contains the superior colliculus that is involved in visual processing. We also examined the cerebellum as a positive control; since it is known to be often severely injured in blasted animals. When we ran the assays, IL-10 in many samples was not reliably detected above background in the brain; and thus, it was dropped from the results and just 10 cytokines were reported. Likewise, for the plasma only CXCL3, ICAM, IL-18, and TIMP-1 showed levels detectable above background. For the mid-brain, cerebellum, and plasma we found that there were no significant differences in cytokine concentrations between placebo and fish oil treated rats at 3 and 7 days following blast exposure; however, in especially the mid-brain, there was an apparent trend for the fish oil group to have slightly lower levels of CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-18, TIMP-1, and TNF- α ($\leq 23\%$). This trend was also seen in the plasma at 3 days out for ICAM-1 and TIMP-1. To examine if any blast-injury effects were present in the brain and plasma, as shown in figure 8, we compared these results to cytokine levels of sham placebo and fish oil treated rats (shams = green; n = 9 and 6 combined). Interestingly, we found for the mid-brain at 3 days out that only TIMP-1 levels were significantly increased above shams

and just in the blasted placebo treated rats (~ 2-fold). The increase in TIMP-1 disappeared at 7 days out, where only IL-18 now showed a significant increase in the placebo treated rats. Cerebellum showed similar results, but also had significantly elevated levels of CXCL2, CXCL3, IL-4, and TNF- α across both of the treatments and days. Likewise, plasma at 3 days out only had significantly elevated IL-18 and TIMP-1 levels. TIMP-1 is a neuroprotective protein that blocks the proteolytic activation of caspases involved in cellular apoptosis. IL-4 is known to beneficially halt activation of M1 macrophages. Thus, elevation of TIMP-1 and IL-4 is a highly desirable effect. In contrast, CXCL2, CXCL3, IL-1- α , IL-18, and TNF- α all are harmful signaling factors, which stimulate immune cell infiltration and activation. Overall, this implies that blast exposure produces marked changes in brain cytokines, but likely the brain visual centers are damaged to smaller degree than other more exposed regions (i.e., cerebellum) or we are not within the window post-blast where the peak changes occur. Also, it is apparent that plasma cytokine changes do not completely reflect those occurring in the brain.

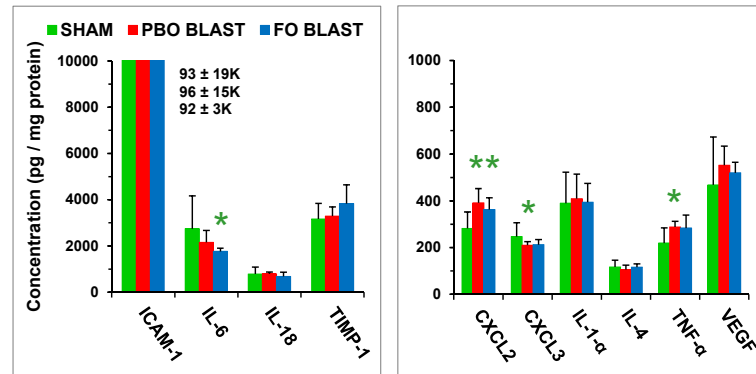
Figure 8: Cytokine levels for brain and plasma of double blasted rats at 3 days post-exposure.



Cerebellum region (3 days)



Cerebellum region (7 days)



Plasma (3 days)

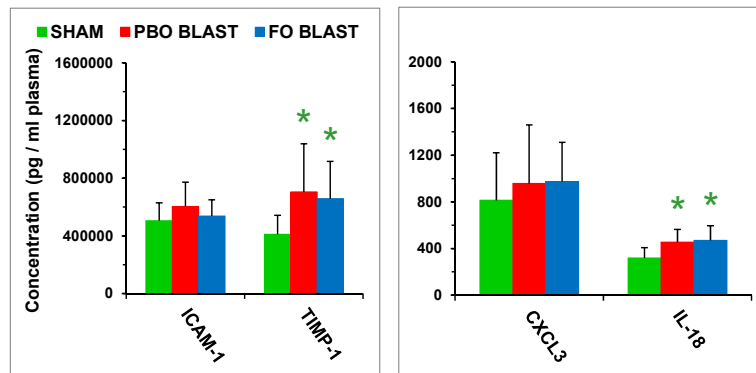


Figure 8. Bar graphs for the mid-brain (top 2 panels), cerebellum (middle 2 panels), and plasma (bottom panel) cytokine levels of placebo versus fish oil treated rats, at 3 and 7 days following double blast exposure, as well as those for sham controls (shams = green, PBO; placebo = red, and FO; fish oil = blue; n = 15, 11, and 10 and 15, 4, and 4, as by day, respectively). For each tissue, the data is broken into two rescaled frames to allow visualization of less abundant cytokines. Tissue concentrations (per mg total protein or ml plasma) for up to 10 cytokines are shown, i.e., CXCL2, CXCL3, ICAM-1, IL-1α, IL-4, IL-6, IL-18, TIMP-1, TNF-α, and VEGF. There were no significant differences detected between dietary treatment groups for blasted animals. *p < 0.05; significant difference between shams and blasted rats, as by t-test.

VII. Histopathology for Eyes and Brains of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Rats from the placebo and fish oil dietary treatment groups will be euthanized at 3, 7, 14, and 28 days post-blast exposure for histopathology assessments of their eyes (retinas) and brain visual centers. Animals are deeply anesthetized by isoflurane inhalation. After surgical opening of the chest and insertion of gravity flow lines in the heart, the rats are perfused transcardially with physiological saline, which results in euthanasia by blood exsanguination, followed by phosphate buffered 4% paraformaldehyde saturated with picric acid (FD Neurotechnologies, Inc) to fix the tissues. Prior to saline perfusion, blood is taken by cardiac puncture via a syringe and placed in chilled EDTA and heparin vacuum collection tubes. Some blood will be centrifuged to obtain the plasma fraction, which is stored frozen at -80°C for future analysis. Whole blood and plasma are used for blood work and cytokine level assessments as previously described.

Eyes and brains are carefully removed from the paraformaldehyde perfused rat heads. To finish the fixation, brains are immersed for up to 6 hours in phosphate buffered 4% paraformaldehyde saturated with picric acid and then washed overnight with buffered 20% sucrose solution. To toughen, the eyes are post-fixed for 6 hours in a mixture of 2% trichloroacetic acid, 2% zinc chloride, 20% isopropanol as made up in paraformaldehyde without picric acid. Post-fixed eyes are washed with phosphate buffered saline followed by 50% ethanol, and then stored in 70% ethanol. Fixed eyes and brains are sectioned, stained, and mounted on microscope slides by FD Neurotechnologies, Inc. Brains are cut into serial coronal sections (30 - 50 µm) through the cerebrum at 11 evenly-spaced positions from stereotaxic coordinates of bregma 1.0 mm to -8.3 mm, as mounted in triplicate. The brain sections target all major visual centers. Eyes are cut as a single horizontal section (5 µm) passing through the central axis of the eye at the optic nerve, as done in triplicate.

Slides are prepared for the tissue sections that are stained with hematoxylin and eosin (H&E; eye and brain) and silver (brain only). H&E stain is reactive towards membrane lipids and proteins, and highlights the cytoplasm and nuclei of neurons as a pink to purple color. Silver stain is highly reactive to proteins, and highlights as a brown to black color the axonal fiber tracts of neurons. Both stains are able to reveal degenerating neurons by differences in morphology and staining intensity. Immunohistochemistry is done on separate eye and brain sections (5 - 15 µm) for the presence of immune cells involved in inflammation that are well known to express the proteins Iba-1 (ionized calcium binding adaptor molecule 1), CD68 (cluster of differentiation 68), and GFAP (glial fibrillary acidic protein), i.e., activated microglia & macrophages, macrophages, and astrocytes, respectively. Sections are exposed to streptavidin-biotin conjugated primary antibodies toward these proteins (DAKO USA, AbD Serotec Bio-Rad, and Wako Chemicals, respectively), generating a dark brown coloration of cells overexpressing them, which have infiltrated and/or become activated within the neuronal tissues. Activation of microglia is also characterized by formation of ball and elongated shapes having diminished fiber

extensions (non-ramified). For the eyes only the retinas are examined, whereas for the brain mainly the optic tracts and superior colliculus are assessed, using an axial microscope having an image capture camera linked to a computer. Histopathology results are expressed as representative images, staining intensities, morphological perturbations, and/or cell body counts.

Results and Conclusions:

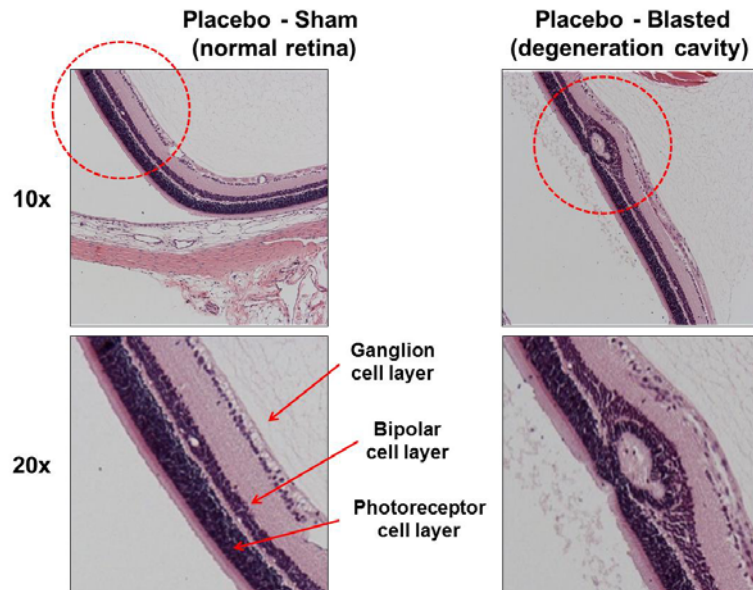
We have done histopathology for eyes (retina) and brains from a total of 3 placebo and 3 fish oil treated rat taken at 7 days post-blast. We also carried out eye and brain histopathology for 1 placebo and 1 fish oil treated sham, as background controls. Microscope slides have been returned and fully assessed for H&E and silver stains and CD68, GFAP, and Iba-1 immunohistochemistry (IHC). We are still waiting for return of eye and brain slides for 1 blasted placebo treated rat collected at 3 days out. Shown in Figure 9 are representative images for eye sections (panels 1, 2, and 3), which are close up shots (10 - 20x magnification) of the right side retinas from rats for the two dietary treatments. Under H&E stain, placebo and fish oil treated rats equally appear to have mild to moderate ongoing retinal degeneration, when compared to shams, which is consistent with the right eye having faced the oncoming blast wave (panels 1 and 2). The left side retinas also had signs of retinal degeneration, but to a lesser degree (images not shown). In support of these results, Iba-1 and GFAP IHC for the right side retinas showed an extreme amount of activated microglia and astrocytes (i.e., Müller cells), respectively, to be present in both treatment groups following blast exposure (panels 2 and 3). Location of the Iba-1 positive microglia within the retina was discerned by weakly counter staining the sections with cresyl violet, which accentuates the photoreceptor and bipolar cell layers (panel 3). Activated microglia cells are clustered near the ganglion cell layer, which has been purposed by others to be most susceptible to damage by blast wave exposure. On the other hand, the activated astrocytes are most concentrated in the photoreceptor layer, which is known to be the most sensitive part of the retina to physical disturbances, e.g., detachments. We also looked at CD68 IHC of the retinas, but found a complete absence of activated macrophages in the shams and blasted rats, which implies infiltration of these immune cells into the tissue has not yet occurred by 7 days post-exposure.

Also, shown in Figure 9, are representative images of the brain sections (panels 4 and 5) for corresponding rats from the two dietary treatments, which are close up shots (10 - 20x magnification) of the left side optic tract brain region. The left optic tract is innervated with the right retina through substantial axonal cross over at the optic chiasm. Under silver stain, both placebo and fish oil treated rats appear to have ongoing axonal fiber tract degeneration in their optic tracts, when compared to shams; however, it does appear to be more severe in the placebo group. The right side optic tracts also had signs of neurodegeneration, but typically to a much lesser degree (images not shown). In support of these results, Iba-1, CD68, and GFAP IHC for the left optic tracts showed robust levels of activated microglia, macrophages, and astrocytes respectively, to be present in both treatment groups following blast exposure (panels 4 and 5). The density of these activated immune cells appears to be nearly equal in the two treatment groups, except Iba-1 positive microglia maybe slightly lower in the optic tracts of the blasted fish oil treated rats. Overall, the brain and retina results agree that neuronal degeneration is present

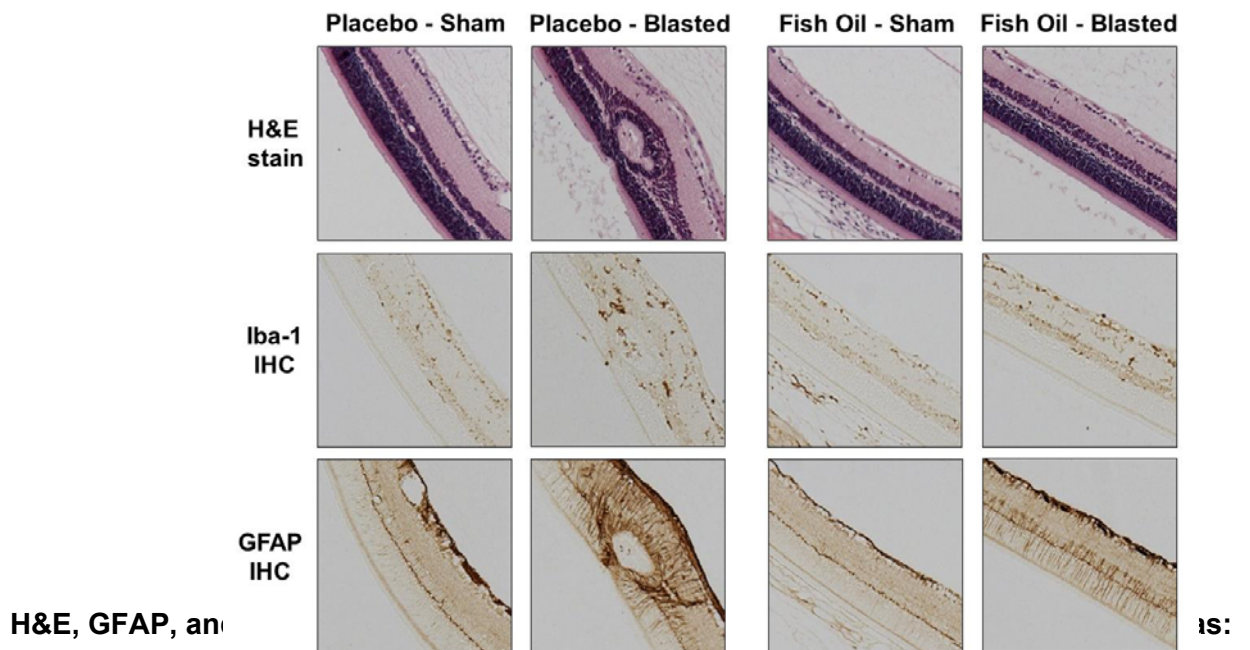
post-blast and dietary omega-3 fatty acids fail to alleviate this.

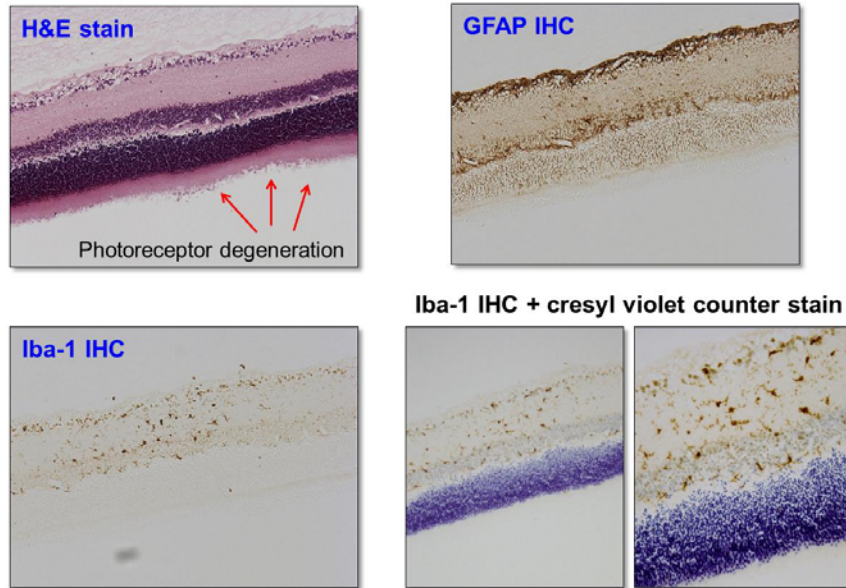
Figure 9: Histopathology for eyes (retinas) and brains of double blasted rats at 7 days post-exposure.

H&E staining of right side retinas for general cell morphology:

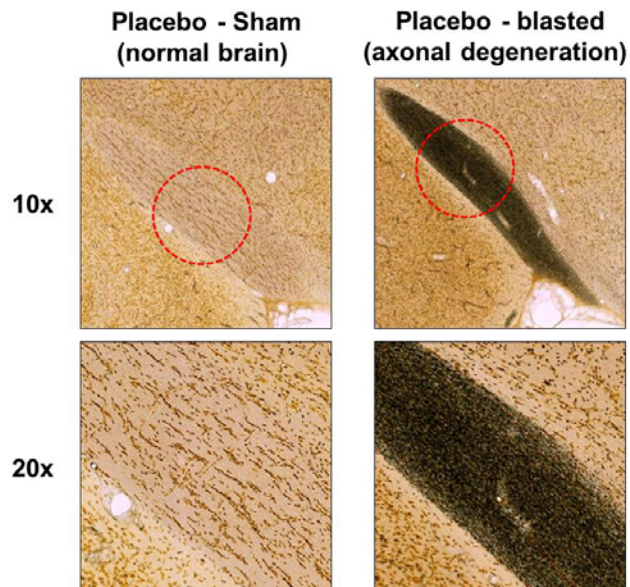


Iba-1 and GFAP IHC of retinas for activated microglia and astrocytes:





Silver staining of left side brain optic tracts for axonal fiber tract degeneration:



Iba-1, CD68, and GFAP IHC of brain optic tracts for activated microglia, macrophages and

astrocytes:

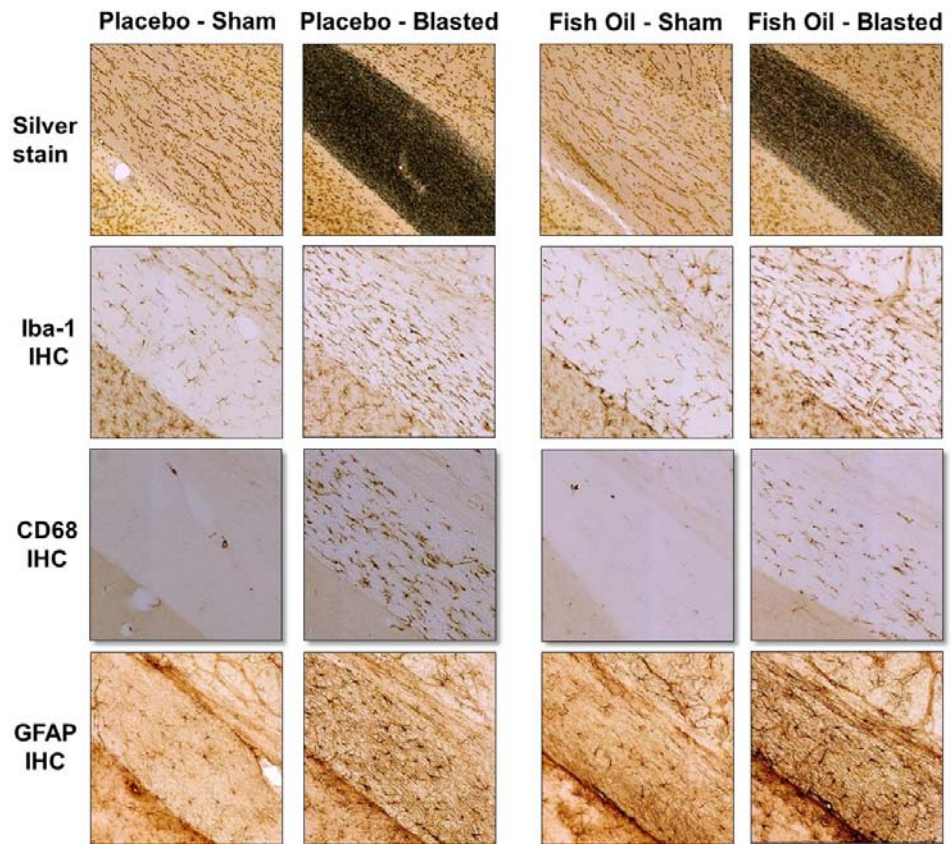


Figure 9. Representative images of retinas from rats for the two dietary treatments. Likewise, representative images are displayed for the left side brain optic tracts from the corresponding animals (panels 4 and 5). H&E and silver stains highlight general cell morphology and axonal fiber tract degeneration, respectively. Iba-1, CD68, and GFAP IHC highlights activated microglia, macrophages, and astrocytes, respectively. Retina and brain optic tract images were taken at 10 - 20x magnification to help reveal cellular features.

VIII. MRI analysis for Eyes and Brains of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Twenty four hours preceding the experimental end points of 3, 7, and 28 days post-blast, rats receive i.v. injections of a ^{19}F -labeled perfluorocarbon-based contrast agent (VSense-580; Celsense, Pittsburgh, PA). The MRI contrast agent is non-toxic, taken up by macrophages as foreign particles, and excess eliminated via hepatic and renal mechanisms. Administration is done by placing the rats under isoflurane anesthesia; inserting a butterfly catheter with 25G needle into the lateral tail vein; and then by syringe pump, infusing the contrast agent emulsion at a dose of 3.3 ml/kg and rate of 0.5 ml/min. At 24 hours later, animals are anesthetized with isoflurane and perfused with saline followed by 4% paraformaldehyde saturated with picric acid. Prior to the perfusions, whole blood is collected via cardiac puncture and frozen away to later determine the background levels of MRI contrast agent. Fixed carcasses are fully sutured shut and sent out to our collaborators at the McGowan Institute's Animal Imaging Center at the University of Pittsburgh (Dr. Hitchens' lab) for ^{19}F and ^1H MRI analysis of the in situ eyes (retina) and

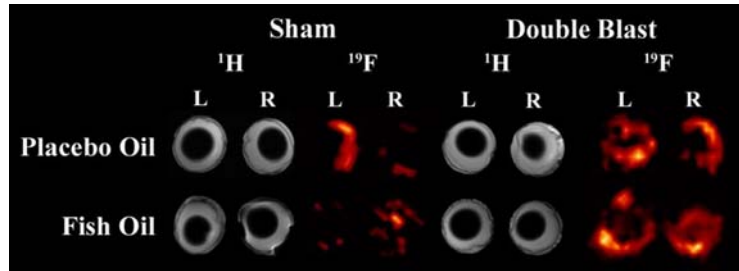
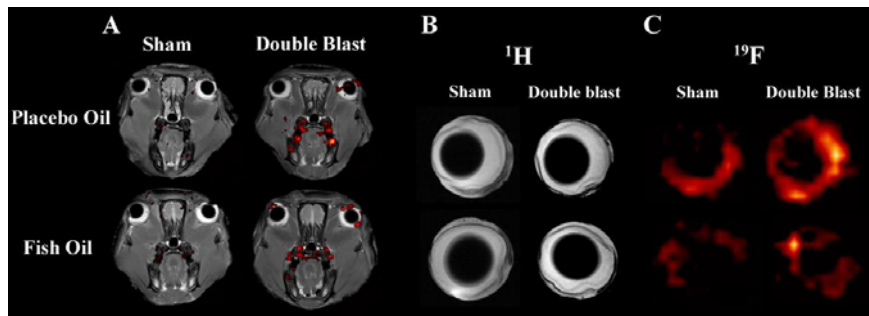
brains. Rats are put through an MRI machine (Bruker, Biospec AVANCE 3; 9.4 Tesla / 21 cm), set to a 2 mm slice thickness and ~ 70 μ m spatial resolution. ^{19}F images are overlaid onto ^1H images to place them into anatomical context and to assess ^{19}F -labeled macrophage accumulation for determining the distribution and severity of neuroinflammation sites. High resolution 3D Magnetic Resonance Microscopy (MRM) is also performed at 11.7 Tesla (≤ 20 μ m resolution) to more finely assess any changes resulting from blast exposure. After imaging, eyes and brains are removed and shipped back to us for histopathology assessments, as previously described.

Results and Conclusions:

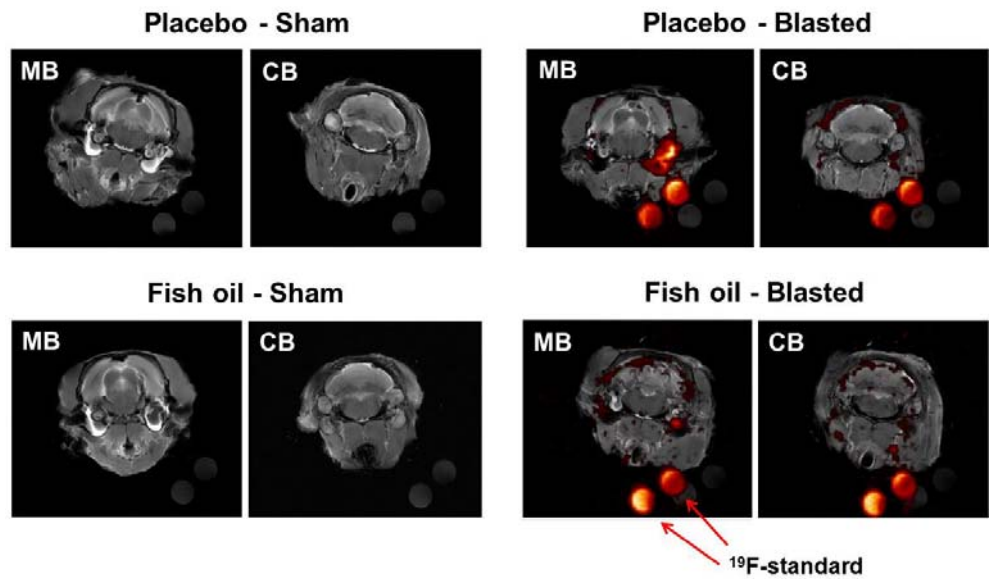
We have submitted to the Animal Imaging Center at the University of Pittsburgh (McGowan Institute) a total of 16 rats for ^1H and ^{19}F MRI analysis of their eyes (retina) and brains, i.e., 4 sham placebo, 4 blasted placebo, 4 sham fish oil, and 4 blasted fish oil treated rats at 3 days post-exposure. Preliminary, film image sets have been returned to us for all of these animals and interpreted with help of the head of the MRI facility. Shown in figure 10 are representative structural anatomy (^1H -MRI) and corresponding macrophage cell tracking (^{19}F -MRI) images for the in situ eyes and brains (i.e., mid-brain and cerebellum planes) of placebo and fish oil treated sham and blasted animals (panels 1 and 2). Composite images from most of the rats done to date are displayed below. While neuronal tissue damage is not discernable on the structural anatomy views at this resolution, the overlays and side by side comparisons with the corresponding ^{19}F scans show marked deposits of macrophages, displayed in “red hot” pseudo-color, are occurring at potential blast induced inflammation sites in the blasted animals compared to shams. For the eyes (panel 1), the blasted rats have extensive macrophage infiltration present, which fills the entire globe space and shows no preference for the right versus left eye, despite the blast impact being focused at the right side of the head. Likewise, macrophage accumulation appears to be relatively the same in both of the blasted treatment groups. Interestingly, on the whole head images (top sub-panel, part A) there appears to be extensive macrophage accretion (i.e., inflammation) in the nares of the rat’s nasal cavity, which maybe slightly less in the fish oil treated animals. Also, shown in Figure 10 are images of the corresponding brains, i.e., mid-brain and cerebellum (panel 2) for the rats from the two dietary treatments. As for the eyes, these brain regions in the blasted rats compared to shams show extensive macrophage infiltration that primarily rings the meninges with some penetration into the grey matter. Again, there is no strong indication that the macrophage accretion is reduced in the fish oil compared to placebo treated animals. The brain images also suggest that macrophages are aggregating near the bottom of the ear canals, which like our findings for the nasal cavity, would suggest that the blast wave is being transmitted down these channels into the skull and brain.

Figure 10: MRI (^1H and ^{19}F) for eyes and brains of double blasted rats at 3 days post-exposure.

MRI based structural anatomy and macrophage cell tracking of eyes:



MRI based structural anatomy and macrophage cell tracking of mid-brain and cerebellum:



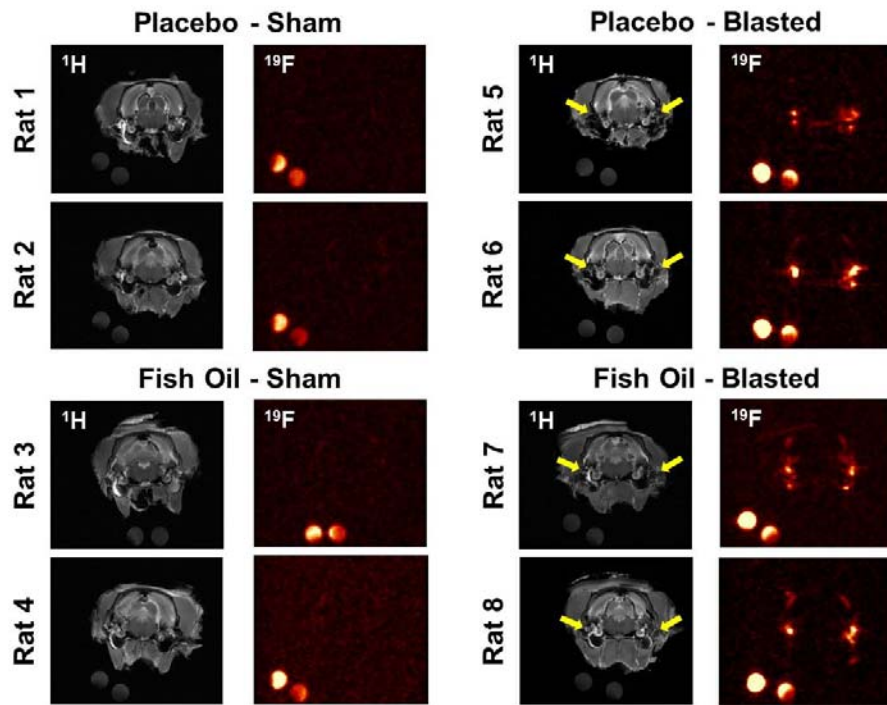


Figure 10. Representative structural anatomy (^1H -MRI) and the corresponding macrophage cell tracking (^{19}F -MRI) images for the in situ eyes and brains (i.e., mid-brain and cerebellum planes) of placebo and fish oil treated sham and blasted animals (panels 1 and 2). For the upper most sub-panel (panel 1), in situ images of head (A) eyes (B & C) of representative sham and blasted rats (house chow fed) are given. The relative amount of ^{19}F signal is also shown by spectroscopy (D). The ^{19}F signal for all images is displayed in “red hot” pseudo-color, which is a sign of macrophage infiltration.

- **What opportunities for training and professional development has the project provided?**

1) Attended the National Capital Area TBI symposium held at the NIH in Bethesda MD, on 4 - 5 April 2016. At the meeting, there were many poster presentations on clinical aspects of visual system injuries in US soldiers after blast wave exposure. I was able to touch base with our local colleagues working in the field of blast induced neurotrauma. I also presented a poster on my initial data from the project at this meeting.

2) Attended the National Neurotrauma Society Symposium held in Lexington KY, on 25 - 30 June 2016. At the meeting there were many talks and poster presentations on treatment of neuronal damage to the retina and brain due to various forms physical injury. Most importantly, I was able to connect with Dr. Tonia S. Rex, from the Vanderbilt University Eye Institute - who models air blast induced ocular injuries in mice, and verify the validity of my retina injury findings with her. I also made connections with Dr. Andrew J. Morris, from the Biomedical Mass Spectrometry Core Laboratory at the University of Kentucky, who specializes in detection of trace lipids in tissues. He is willing to screen (without charge) my rat retina and brain

samples for changes in levels of anti-inflammatory metabolites of omega-3 fatty acids, e.g. neuroprotectins. I attended several workshops focusing on animal models of neurotrauma, neuroprotective drug discovery, and brain histopathology techniques. I also presented a poster on my updated data from the project at this meeting.

3) Attended the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando, FL, on 15 - 18, August 2016. At the meeting, there were many talks and poster presentations on aspects of traumatic brain injuries, including those to the visual system, in animal models and US soldiers after blast wave exposure. The focus of this meeting was broad, covering blast physics, injury pathologies / diagnosis, and prevention / treatment strategies. I was able to touch base with our local, national, and international colleagues in the field of blast induced neurotrauma. I also presented a poster on my updated data from the project at this meeting.

- **How were the results disseminated to communities of interest?**

At the three conferences listed above, I presented the data from this study in poster format at single day sessions that were open to all attendees, including representatives from the scientific industry sectors (e.g. lab supply companies).

- **What do you plan to do during the next reporting period to accomplish the goals?**

I plan on bringing in at least 12 - 18 rats per month and feed them up for one month on the omega-3 polyunsaturated deficient and enriched diets (gavaged daily with placebo and fish oils, respectively), as to prepare them for double blast wave exposure followed by filling in the retina and brain assessments at 3 and 7 days out (i.e., acute effects), before we begin to tackle the chronic period of 28 days out. For the upcoming 1st quarter period the most feasible outcome measures for us to perform will continue to be electroretinography (ERG), visual acuity task, and fresh tissue collections for cytokine / chemokine levels by immunoassay arrays. We will especially focus on filling in more animals at 7 days post-blast. The ERG exams will include standard full field flash for retinal signaling to light and possibly visual evoked brain potentials (VEBP) for retinal plus brain visual processing center function. While we have not yet established capabilities to do pattern ERG testing with our instrument for specific detection of retinal ganglion signaling, we are currently in contact with manufacturer to purchase or lease the necessary accessories to do this, i.e., pattern stimulator video screens. MRI analysis (structural anatomy and macrophage cell tracking) will also be continued with our outside collaborators at the Animal Imaging Center of the University of Pittsburgh. We will focus on filling in animals at 3 and 7 days post-blast and send out their returned eyes (retinas) and brains to a local company (FD Neurotechnologies) for histopathology to detect the presence of activated immune cells and neuronal cell death, i.e., CD68, GFAP, and Iba-1 immunohistochemistry (astrocyte, microglia, and macrophage biomarkers) and H&E and silver staining (morphological

perturbations and axonal fiber tract degeneration). We will hold off doing MRI analysis on rats at 28 days post-blast until much later in the study, due to the extreme time investment involved in maintaining the animals. Overall, the cytokine assays and histopathology are already providing good information on potential drug targets and their therapeutic windows as well as the efficacy of omega-3 fatty acid supplementation to alleviate blast induced visual system damage. We have already have targets and windows in mind, i.e., acutely administered agonists of TIMP-1 (e.g. genipin), to try in our other ongoing projects looking at neuroprotective drugs in blast wave exposed rats.

- **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

There is nothing to report, since we are still in the middle stage of the project and have only provided our preliminary findings to other labs in the field of blast induced neurotrauma, with a caution that the conclusions could markedly change as our group sizes and post-injury time points increase. These other labs, however, have shown interest in some of the novel methodologies behind our work, i.e., blast wave simulation by shock tube and macrophage cell tracking by MRI; and thus, want to incorporate these techniques into their studies with advisement from us.

- **What was the impact on other disciplines?**

There is nothing to report, since we are primarily providing our preliminary findings to only other labs in the field of blast induced neurotrauma. Other disciplines within science have so far shown very little appreciable interest in our current work. This is mainly a result of our negative findings so far for a benefit of dietary omega-3 fatty acids to heal neuronal injuries independent of the insult type and extent, which is widely thought to be the case.

- **What was the impact on technology transfer?**

There is nothing to report, since this project does not involve development of a new technology.

- **What was the impact on society beyond science and technology?**

There is nothing to report, since we are not officially releasing our preliminary findings to the general public at this time. Our data is not substantiated enough to provide trustworthy recommendations on the use of omega-3 fatty acid supplements to treat blast induced neurotrauma to the visual system in human populations. If our results continue in the present direction of a slight efficacy, if any, they may be eventually used by the medical profession to warn patients not to rely on increased intake of omega-3 fatty acids as sole treatment approach. Dietary omega-3 fatty acids have been a growing nutritional fad for the prevention of many neurological

afflictions. Ultimately, it may prove to be an adjunct supportive therapy at best for this specific type of insult to the retina and brain.

- **CHANGES/PROBLEMS::**

- **Changes in approach and reasons for change**

There are no changes in experimental approach as found in the approved animal protocol during this reporting period; however, it significantly deviates from the original grant proposal as follows:

1) Number of animals brought in for each experiment has been increased to 12 - 18 rats, in order to compensate for the large number of accidental animal deaths (i.e., up to 33%) incurred during blasting of the rats. These losses for the most part are unavoidable, since necropsy has shown that poly-trauma complications, i.e., brain, heart, lung, and/or liver damage, to be the primary cause of sudden death in the blasted rats. We have found that the only way to diminish this is to make sure the animal is fully secure in the holder during blasting to prevent additional losses from grave injuries due to movement and striking of objects as the blast wave passes by.

2) Cytokine levels in the plasma, brain, and retina of blasted rats are being measured by multiplex immunoassay arrays (Luminex; R&D Systems, Inc). These arrays can determine the simultaneous concentrations of up to 17 rat specific factors, are rapid to complete (< 9 hours), run 96 samples at once, and are ultrasensitive down to the pico-molar range. We selected 11 of the available factors to assay, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-10, IL-18, TIMP-1, TNF- α , and VEGF. Originally, we had planned to analyze the samples using immunoassay micro-blot arrays (R&D Systems, Inc), which simultaneously detect 29 distinct types of cytokines / chemokines; however, while these arrays are lower in cost, they provide only qualitative measurement of any changes.

3) We have over focused on doing blasted animals under both dietary treatments groups for ERG, visual acuity, and cytokine immunoassay array analysis at 3 and 7 days out. Work up of blasted animals for MRI of the eyes (retina) and brain and subsequent histopathology had taken a back seat to this, due to our collaborator for the MRI analysis, Dr. T. Kevin Hitchens, moving his lab from Carnegie Mellon University to the University of Pittsburgh. He now director of the McGowan Institute's Animal Imaging Center, which is added advantage for supporting this project. His new lab was fully opened in June 2016. We have recently carried out MRI analysis on 4 each of shams and blasted rats for the two dietary treatment groups (16 rats total) at 3 days post-exposure. Even though, shams were to be done later on in the statement of work, he requested that their MRI analysis be done now for determination of ^{19}F -labeled contrast agent background signals. He has also requested that ERG recordings not be done on these rats to prevent accidental injury artifacts to the eyes and not interfere with the timing of the contrast agent administration; thus, lowering our anticipated accumulation of this data.

4) We are still awaiting return of the eyes and brains from the MRI analyzed animals for histopathology. Dr. Hitchens, however, has warned us that the eyes and brains may not be suitable for histopathology, due to over fixation in paraformaldehyde and heating during the high magnetic field MRI scans. In the meantime, we have carried out histopathology on eyes (retina) and brains of 1 sham and 3 blasted rats for each of the two dietary groups at 7 days out, as practice for the CD68, Iba-1, and GFAP immunohistochemistry (IHC) to detect activated macrophages, microglia, and astrocytes in the tissues. We can continue to do histopathology on animals at 3 and 7 days post-blast that are not designated for MRI analysis, since we have accumulated plenty of fresh tissue samples to fill in the cytokine determinations at these time points.

5) While we had purposed it as part of the histopathology, our pilot attempts at eye and brain Glut-5 (glucose transporter 5) IHC for activated microglia has shown an extreme amount of non-specific antibody binding to neurons in our rat brain sections (data not shown). We are exploring the possibility of trying antibodies from a different lot or company for this biomarker or dropping it altogether as redundant with the Iba-1 IHC (activated macrophages and microglia) and MRI (macrophages alone) outcomes. Likewise our CD68 IHC on eye sections has suggested that activated macrophages are completely absent in the retinas of our blasted rats, when examined at 3 days out (data not shown). Our MRI analysis results, however, show there to be a massive infiltration of macrophages into the eyes for the two dietary- treatment groups by 3 days post-blast. It is possible that the antibody used for the CD68 IHC is poorly reactive towards macrophages in the eye sections, due to altered epitope factors, which would suggest trying another primary antibody isotype. It is known, however, that CD68 expression in macrophages can be tissue and/or injury specific, so this biomarker may not be useful and another will have to be considered.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

1) We had purposed doing pattern (p) and visual evoked brain potential (VEBP) ERG for determining retinal ganglion cell / optic nerve and brain visual center deficits in the blasted rats. Due to time and equipment problems, we have only analyzed the basic retinal signaling function of the animals, using full field flash ERG, for the two dietary treatment groups at 3 and 7 days out. The VEBP-ERG will be continued to be delayed due to the special surgical efforts required to implant recording screws in the animal's skull over the occipital cortex. Due to the invasiveness of this procedure only a small set of animals can be done, perhaps just at 7 days out, which in other projects seems to be near the peak of retinal signaling deficits. We have done practice VEBP recordings, with limited success, on rats under a different project. It appears that positioning of the screws and lack of contact with muscle tissue is extremely important to obtain a good brain signal after light stimulus of the eyes. We plan to put in an amendment to the animal protocol to routinely do the skull screw implantations on our rats. We are also working with the WRAIR Veterinary Medicine staff to improve the health impact of the procedure on the rats, i.e., a large head wound with exposed screw heads.

While the p-ERG recordings are relatively simple to do, our current system lacks the pattern stimulator video screens (i.e., an alternating checker board) necessary to carry out this test for retinal ganglion cell response. We incorrectly thought the instrument's current flash lab already had this capability. We are having a representative from the company (Diagnosys, LLC) come out to our lab in the next quarterly report period to demonstrate the pattern stimulators to us and present a price quote for purchase or leasing them. At the same time, they will provide us with advice and software upgrades, i.e., band pass filters to eliminate skeletal muscle contraction signals, for doing VEBP recordings.

2) The project continues to fall outside of the main stream mission of the WRAIR Center for Military Psychiatry and Neuroscience Research, whose primary focus is on characterization and treatment of traumatic brain injuries alone. Thus, there is less drive for committing our institute's resources to studies involving peripheral neurosensory deficits (i.e., vision loss). There was also a concern that the project may compete with Army organizations having extensive ocular trauma task areas (e.g., USAISR, San Antonio, TX). Our previous center director, COL Maurice L. Sipos, was very adamant about me considering these points; however, we have a newly appointed center director, LTC (P) Jeffery Thomas, who is more receptive to my eye research projects. His desire is that I continue my efforts with a sensible commitment and pursuit of additional funding. Likewise, we have a new institute commander, COL Deborah L. Whitmer, who in the past has engaged in eye research for the military, i.e., clinical diagnosis of laser injuries to the retina, and is interested in supporting my research. I plan to make an effort to consult more with both LTC Thomas and COL Whitmer regarding the scope and future directions of my studies.

- **Changes that had a significant impact on expenditures**

1) We have greatly increased the group sizes of animals than originally purposed for each treatment and associated outcome measures. This was necessary to appropriately power the study for detecting statistically significant differences between groups. Additional animal and supply costs will be covered by internal funds (USAMRMC-MOMRP) awarded to our lab chief Dr. Joseph B. Long.

2) We are using different immunoassay arrays than originally purposed to measure the cytokine / chemokine levels in plasma, brain, and retina from blast wave exposed rats. These new arrays are fully quantitative micro-titer plate style, as opposed to qualitative micro-blots, but are 3 times more expensive per kit (i.e., \$500 vs. \$1,500); however, in the long run they are more cost effective for the amount of samples that we have to do (i.e., 5 strip blots vs. a 96 well plate).

3) Currently, we have submitted only a limited number of eye and brain samples for histopathology (microscope slides) to the local company FD Neurotechnologies, due to this being an outcome measure done in conjunction (i.e., afterwards) with the MRI analysis of the rats (see sections above).

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- **Significant changes in use or care of human subjects**

The project does not involve the use of human subjects.

- **Significant changes in use or care of vertebrate animals.**

There is nothing to report; since through weekly monitoring of their body weights and blood glucose levels we found that there is no concern for carrying out food restriction to control obesity or metabolic syndrome (pre-diabetes) in the rats.

- **Significant changes in use of biohazards and/or select agents**

There is nothing to report, since this project does not involve the use of marked biohazards and/or select agents (e.g., infectious diseases and toxins / poisons). Syringe needles for animal injections and paraformaldehyde for tissue fixation are the only potential biohazards (low level) that are found in our experiments.

- **PRODUCTS:**

There is nothing to report, since this project does not entail development of a product.

- **Publications, conference papers, and presentations**

1) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the National Capital Area TBI symposium held at the NIH in Bethesda MD, on 4 - 5 April 2016. A copy of the abstract and poster is attached to the annual report.

2) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the National Neurotrauma Society Symposium held in Lexington KY, on 25 - 30 June 2016. A copy of the abstract and poster is attached to the annual report.

3) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando, FL, on 15 - 18, August 2016. A copy of the abstract and poster is attached to the annual report.

- **Journal publications.**

There is nothing to report.

- **Books or other non-periodical, one-time publications.**

There is nothing to report.

- **Other publications, conference papers, and presentations.**

There is nothing to report.

- **Website(s) or other Internet site(s)**

There is nothing to report.

- **Technologies or techniques**

There is nothing to report, since this project does not entail technology or technique development.

- **Inventions, patent applications, and/or licenses**

There is nothing to report, since this project does not entail invention development.

- **Other Products**

There is nothing to report.

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	James C. DeMar, Ph.D.
Project Role:	Principle Investigator - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	12
Contribution to Project:	Directed and helped technicians execute all reported experiments.
Funding Support:	Geneva Foundation contractor – WRAIR

Name:	Joseph B. Long, Ph.D.
Project Role:	Co-Investigator – WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown

Nearest person month worked:	3
Contribution to Project:	Management of personnel and budget for project. Consultant for results
Funding Support:	Civilian government employee – WRAIR

Name:	T. Kevin Hitchens, Ph.D.
Project Role:	Co-Investigator - University of Pittsburgh, McGowan Institute
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	4
Contribution to Project:	Directed MRI analysis of rats; data interpretation
Funding Support:	Faculty - University of Pittsburgh

Name:	Lesley M. Foley
Project Role:	Technician - University of Pittsburgh, McGowan Institute
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	4
Contribution to Project:	MRI analysis of rats; data preparation and presentation
Funding Support:	Faculty - University of Pittsburgh

Name:	John G. Rosenberger
Project Role:	Technician - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	8
Contribution to Project:	Feeding and gavaging (fish oil), body weight and blood glucose measurements, fresh tissue collections, MRI contrast agent infusions of rats. Data entry / analysis.
Funding Support:	Clinical Research Management contractor

Name:	Andrew B. Batuure
Project Role:	Technician - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	10
Contribution to Project:	Visual acuity testing, ERG exams, cytokine immunoassay arrays, fresh tissue

	collections, and MRI contrast agent infusions of rats. Data entry / analysis.
Funding Support:	Clinical Research Management contractor

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

COL Thomas G. Oliver, M.D. (Co-PI; Walter Reed Army Hospital) and Patrick Kochanek, Ph.D. (consultant, Carnegie Mellon University) have not make significant work contributions to the project at this time.

- **What other organizations were involved as partners?**

We have utilized the services of our outside collaborators at the University of Pittsburgh, McGowan Institute's Animal Imaging Center (Dr. T. Kevin Hitchens' lab) for the MRI analyses of our animals and the local company FD Neurotechnologies (Dr. Fu Du; Ellicott City, MD) for histopathology processing (microscope slides) of rat eyes (retinas) and brains. We have active CRADAs in place for the work done with both of these organizations.

- **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

There is nothing additional to report, the efforts by our collaborators at the University of Pittsburgh (Dr. T. Kevin Hitchens' lab) have been extensively detailed in the sections above.

- **QUAD CHARTS:**

An updated Quad Chart is attached.

- **APPENDICES:**

Attached are copies of abstracts and posters presented for the project at three national conferences.

Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers, as a Quest for Therapeutic Drug Targets, in a Rat Model of Blast Over Pressure Wave Exposure. Focus area: Mitigation and treatment of traumatic injuries, war-related injuries, and diseases to ocular structures and the visual system. Funding Opportunity Number: W81XWH-13-CRMRP-VRP-HDA



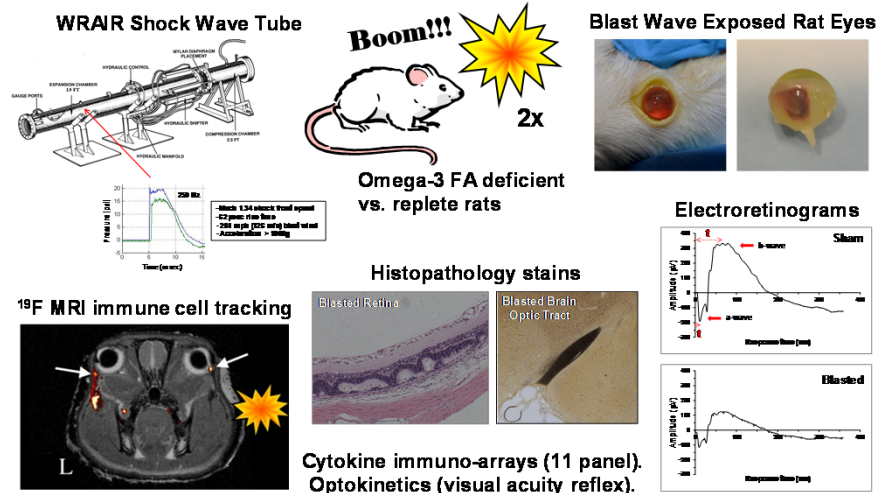
PI: James C. DeMar, Jr., Ph.D. Org: Walter Reed Army Institute of Research / The Geneva Foundation Award Amount: \$249,998

Study/Product Aim(s)

- Blast overpressure (BOP) is a leading cause of vision loss in US soldiers, due to closed eye injuries (43% incidence with 26% involving the retina).
- Very few animal studies have characterized BOP induced visual system injuries or looked for drug therapeutics.
- We hypothesize that immune cell mediated processes play a central role in promoting neuronal cell death in the blasted retina and brain.
- Our objective is to monitor nature and timing of inflammatory processes in retina and brain visual centers of BOP exposed rats to discern drug targets and therapeutic windows. Dietary omega-3 fatty acid impact will be studied.

Approach

(1) Rats on an omega-3 fatty acid deficient or enriched diet exposed twice to blast waves (20 psi) using a shock tube. (2) At 3, 7, 14, and 28 days post-blast, inject with ^{19}F MRI tracer to in vivo label macrophages, and then perfusion fix. (3) Send to Univ. Pittsburgh for anatomical and cell tracking MRI of eyes and brain. (4) Histopathology of fixed eyes and brains for morphology, axonal degeneration, and activated immune cells. (5) Cytokine immunoassay arrays on fresh plasma, retinas, and brains. (6) Electroretinogram (retinal signaling) and optokinetic (visual acuity) tests done on all rats.



Accomplishments: 56 rats, fed omega-3 deficient or enriched diets, were blasted and visual acuity, ERG, cytokine, histopathology, and MRI tests done at 3 & 7 days out.

Timeline and Cost

Activities	CY	14	15	16	17
Write animal protocol and submit to WRAIR-IACUC and USAMRMC-ACURO for approval.					
Complete 3 and 7 day post-blast time points in rats for all outcome measures.					
Complete 14 and 28 day post-blast time points in rats for all outcome measures.					
Wrap up data and then write final reports and publications.					
Estimated Budget (\$K)		\$125K	\$125K	\$000	\$000

1 year, no cost extension approved

Updated: 9 November, 2016

Goals/Milestones

CY14 Goals – Initiate study; animal testing:

- Obtain a WRAIR-IACUC and USAMRMC-ACURO approved animal use protocol. Purchase rats, diets, and immunoassay array kits. Train on instrumentation.
- Blast rats, which are kept on omega-3 fatty acid deficient or enriched diets, and at 3 and 7 days post-injury do all outcome measures. (n = 4 - 22, each).
- Non-blasted rats will also be analyzed in same manner at 28 days out.

CY15 Goals – Complete study; drug targets and windows identified:

- Blast rats, kept on diets, and at 14 and 28 days post-injury do all outcome measures as above (n = 4 - 22, each). One year no cost extension needed?

Comments/Challenges/Issues/Concerns

- We recently finished MRI analysis of 16 blasted rats at 3 days out; these results in particular suggest omega-3s have little efficacy towards treating neuronal injuries.
- We are most behind number wise for histopathology of blasted eyes and brains.
- We need to fill in the 7 day post-blast time point, but also start 14 or 28 days out.

Budget Expenditure to Date

Projected Expenditure: \$ 166,665 Actual Expenditure: \$77,555.94

Characterization of Inflammatory Processes in the Visual Systems of Rats Induced by Exposure to Primary Blast Waves

James DeMar, PhD¹, John Rosenberger, BS¹, Andrew Batuure, BS¹, Donna Wilder, BS¹, Meghan Mccuiston, BS¹, Patrick Kochanek, MD², Lesley Foley, BS³, and Kevin Hitchens, PhD³, and Joseph Long, PhD¹.

¹Walter Reed Army Institute of Research, Center for Military Psychiatry and Neuroscience Research, Silver Spring, MD.

²University of Pittsburgh School of Medicine, Safar Center for Resuscitation Research, Pittsburgh, PA.

³University of Pittsburgh, Animal Imaging Center, Pittsburgh, PA.

BACKGROUND: Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. **METHODS:** Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; 19F-perfluorocarbon contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (Iba-1 and GFAP immunohistochemistry), and cytokine level (multiplex immunoassay arrays) outcome measures. **RESULTS:** Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus so far shown slight, if any, ability to alleviate these acute injury events. **CONCLUSIONS:** In rats, blast wave exposure causes marked neuronal cell damage to the visual

system (retina and brain) that is associated with multi-faceted inflammatory processes. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not readily alleviate these acute injury events. Chronic events, e.g. neuronal cell repair, may be more amenable to other functions of omega-3 fatty acids, such as membrane structural restoration. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data that will lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army. SUPPORT: Funded by DoD grants from the MOMRP and USAMRMC / CDMRP, #: W81XWH-14-2-0178.

Characterization of Inflammatory Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

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¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Spring, MD; ²Safar Center for Resuscitation Research and ³Animal Imaging Center, University of Pittsburgh, Pittsburgh, PA.



ABSTRACT

BACKGROUND: Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory (e.g. a diet enriched in omega-3 polyunsaturated fatty acids) on blast injury vulnerability. **METHODS:** Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; ¹⁹F-perfluorocarbon contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (Iba-1 and GFAP immunohistochemistry), and cytokine level (multiplex immunoassay arrays) outcome measures. **RESULTS:** Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus far shown little, if any, ability to alleviate these acute injury events. **CONCLUSIONS:** In rats, blast wave exposure causes marked neuronal cell damage to the visual system (retina and brain) that is associated with multi-faceted inflammatory processes. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not readily alleviate these acute injury events. Chronic events, e.g. neuronal cell repair, may be more amenable to other functions of omega-3 fatty acids, such as membrane structural restoration. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data that will lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178.

BACKGROUND

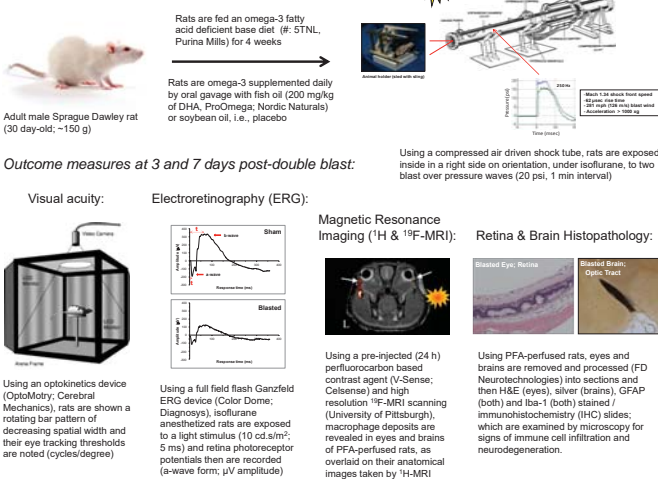
Blast exposure has caused since 2008 about 280,000 cases of traumatic brain injury in U.S. Soldiers, often with symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013). Of these patients, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011). While soldiers wear protective goggles, they still can suffer eye injuries, e.g. blast wave penetration (Weichel, 2006). Few animal studies have tried to characterize visual system injuries as generated by high fidelity blast waves and/or have evaluated drug treatments (review by DeMar, 2016). Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010). Dietary omega-3 polyunsaturated fatty acids are converted to molecules, e.g. neuroprotectins and resolvins, which can suppress immune cell activation; and thus, may represent a practical therapeutic intervention (Serhan, 2008, 2011, 2012).

References: Capo-Aponte, 2012. Mil. Med. 177: 804-813; Cockerham, 2011. N. Engl. J. Med. 364: 2172-2173; DeMar, 2016. Front. Neurol. 7: 59 (1-12); Lemke, 2013. JAMA Ophthalmol. 131: 1602-1609; and Serhan, 2008, 2010, 2011, & 2012. British J. Pharm. 155: 5200-5215, Am. J. Pathol. 177: 1578-1591, Curr. Top. Med. Chem. 11: 629-647, & Chem. Rev. 111(10) 5932-5943; and Weichel, 2006. Curr. Opin. Ophthalmol. 19: 519-525.

In our study, rats were maintained on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves in a shock tube; and the resulting visual dysfunction and underlying neuroinflammation and neurodegeneration in the brain and retina was characterized by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Cytokine levels and Magnetic Resonance Imaging (MRI) based macrophage tracking.
- 3) Histopathology (H&E and silver stains; and GFAP & Iba-1 IHC).

METHODS



Brain cytokines:

Using terminal exsanguination, under isoflurane anesthesia, rats were sacrificed and whole brains collected and frozen. Thawed tissue was dissected into mid-brain containing the superior colliculus, i.e., a visual processing center, and the cerebellum as a known blast-injury site control. Cytokine levels of brain regions were determined, using protein lysates and immunoassay arrays (rat specific 10-plex; Luminox; R&D Systems). Values for cytokines were expressed as concentrations (pg/mg), after normalization against total brain protein (BCA assay; Thermo-Scientific).

DATA and RESULTS

Double blast impact on visual acuities and electroretinography (ERG) at 3 and 7 days post-exposure; both treatment groups show similar deficits in spatial resolution and retinal signaling function:

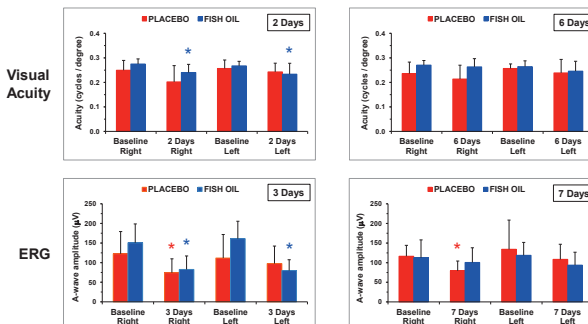


Figure 1. Visual acuities (cycles/degree) and ERG amplitudes (µV; a-wave) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 2-, 3 and 6-7 days post-blast. Group sizes: n = 11 and 12 and n = 7 and 11, respectively. *p < 0.05; significant difference from baseline, as by t-test.

Double blast impact on brain cytokines at 3 days post-exposure; both treatment groups show a similar increase in regional brain levels of specific cytokines:

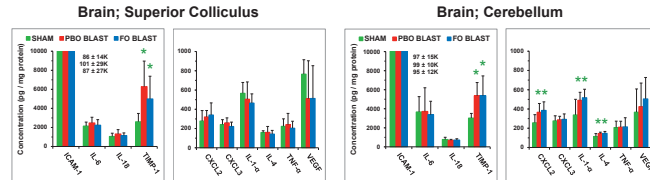


Figure 2. Brain cytokine levels (pg/mg) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, at 3 days post-blast. Shams were non-injured and not given omega-3 fatty acids. Group sizes: n = 7, 8, and 7 each, respectively, for superior colliculus and cerebellum regions. *p < 0.05; significant difference from shams (non-blasted), as by t-test. PBO = Placebo and FO = Fish Oil treatments. 10 cytokines were assayed.

Double blast impact on eye (in situ) ¹⁹F-MRI based macrophage tracking at 3 days post-exposure; both treatment groups show a similar presence of immune cell infiltration in the injured eyes:

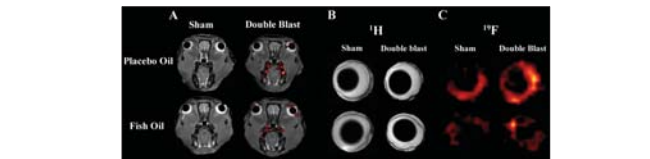


Figure 3. ¹H-MRI anatomical and ¹⁹F-MRI macrophage tracking analysis of rats fed omega-3 deficient (placebo) and enriched (fish oil) diets, for shams or at 3 days post-blast. In situ images of head (A) and merged eyes (B, C) of four representative animals. ¹⁹F signal is shown in red pseudo color (A, C).

Double blast impact on eye (retina) and brain (optic tract) histopathology at 7 days post-exposure; both treatment groups show a similar degree of neurodegeneration and immune cell activation:

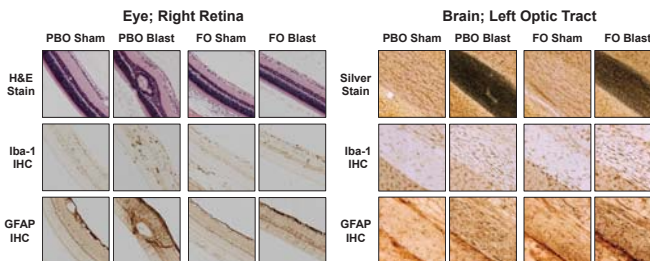


Figure 4. Histopathology of eyes (right retina) and corresponding brains (left optic tract) from four representative rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, for shams or at 7 days post-blast. PBO = Placebo and FO = Fish Oil treatments. Magnification is 10-20x.

CONCLUSIONS

Our current findings with visual acuity and electroretinography (ERG) testing in rats reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days). These deficits are accompanied by neurodegeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines, as obtained by immunoassay arrays, magnetic resonance imaging (MRI), and histopathology. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown little, if any, ability to alleviate these injury events, despite having potent anti-inflammatory properties. Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals.

Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

¹James C. DeMar, ¹John G. Rosenberger, ¹Andrew B. Batuure, ¹Donna M. Wilder, ¹Meghan A. Mccuiston, ²Lesley M. Foley, ²Patrick M. Kochanek, and ²T. Kevin Hitchens, and ¹Joseph B. Long.

¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ²University of Pittsburgh, Pittsburgh, PA 15261.

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; 18F-contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (immunohistochemistry), and cytokine level (immunoassay arrays) outcome measures. Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 3 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus so far shown little ability to alleviate these acute injury events. Overall, our mission is to provide data that will ultimately lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: This work is supported by DoD grant awards from the MOMRP and USAMRMC / CDMRP (#: W81XWH-14-2-0178).



Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

^{1,2}James C. DeMar, Ph.D., ¹John C. Rosenberger, ¹Andrew B. Batuure, ¹Daniel J. Thadieu, ^{1,2}Donna M. Wilder, ¹Meghan A. Mccuistion, ³Lesley M. Foley, ³Patrick M. Kochanek, and ³T. Kevin Hitchens, and ¹Joseph B. Long.

¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ²As Contracted Through The Geneva Foundation, Tacoma, WA 98402; ³McGowan Institute, Animal Imaging Center, University of Pittsburgh, Pittsburgh, PA 15203



Background

Blast injury has emerged as arguably the greatest threat to Warfighters in current campaigns, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from shock waves. Despite the serious disability that vision loss represents, there are few animal studies that rigorously assessed blast wave injuries to the visual system. These studies often suffer from poor simulation of blast injuries or are limited in scope of outcome measures. None have studied the interplay of damage to the retina and brain. Thus, there is an urgent need to do advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role in dictating the extent of neuronal cell death in retina and brain following blast. Using adult rats exposed to shock tube generated blast waves, our objective is to longitudinally monitor up to 28 days the nature and timing of immune cell guided inflammatory processes in the injured retina and brain as to discern potential drug targets and therapeutic windows. We are also examining the impact of interventions known to be anti-inflammatory, i.e., giving the rats a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Overall, our mission is to provide data that will ultimately lead to discovery of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grant awards from the MOMP and USAMRMC / CDMRP/ Vision Research Program #: W81XWH-14-2-0178.

Introduction

□ Blast injuries have resulted since 2008 in more than 280,000 cases of traumatic brain injury in U.S. Soldiers, with frequent symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013).

□ While brain visual centers can be affected, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011).

□ Soldiers are issued protective goggles but still suffer eye injuries, such as from blast wave penetration (Weichel, 2008).

□ Few animal studies have tried to characterize visual system injuries as generated by rigorously simulated blast waves; and only three have evaluated drug treatments (review by DeMar, 2016).

□ Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010).

□ Metabolites of dietary omega-3 polyunsaturated fatty acids can suppress immune cell activation (Serhan, 2008, 2011, 2012).

References:
Capo-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
DeMar et al., 2016; Front. Neurol. Accepted pending revision.
Lemke et al., 2013; JAMA Ophthalmol. 131(12): 1602-1609.
Serhan et al., 2008, 2010, 2011, & 2012; British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177(4) 1576-1591, Curr. Top. Med. Chem. 11(6) 629-647, & Chem. Rev. 111(10) 5922-5943.
Weichel et al., 2008; Curr. Opin. Ophthalmol. 19(6): 519-525.

Aim of Study

In rats raised on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves, characterize up to 28 days out the visual dysfunction and underlying neuro-inflammation / degeneration due to retina and brain injuries, as by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Bloodwork, tissue fatty acids and cytokines, and Magnetic Resonance Imaging (MRI) based immune cell tracking.
- 3) Histopathology (H&E and silver stains; Iba-1 and GFAP IHC).

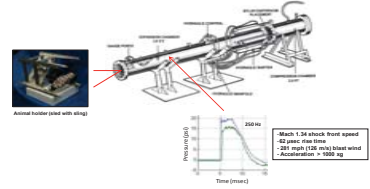
Materials and Methods

Animals and Dietary Manipulations:

Adult male Sprague Dawley rats (30 d-old) are fed for 4 weeks a chow devoid of long chain omega-3 polyunsaturated fatty acids (#: 5TLN; Purina Mills). Some animals are omega-3 supplemented with fish oil (ProOmega; Nordic Naturals), daily by gavage to provide 200 and 273 mg/kg of DHA and EPA. Placebo controls are given an equal volume of soybean oil.

Simulation of Primary Blast Wave Injuries:

□ Rats are exposed under isoflurane anesthesia to two blast over pressure waves, i.e., double blast (20 psi; 1 min interval), in a right-side on position, using a compressed air driven shock tube.



Visual Acuity (Optokinetics):

Using an optokinetics device (Optomotry; Cerebral-Mechanics), rats are put on a pedestal in a chamber, where a rotating bar pattern is shown on four LCD monitors. Rotation speed is increased stepwise to narrow the bar's width; and visual acuity threshold (cycles/degree) is found, when animal ceases head-eye tracking movements (nystagmus). Separate eye acuity are determined by driving the rotation in opposite directions.

Electroretinography (ERG):

Rats are dark adapted for 16 h. Using a full field flash Ganzfeld ERG device (Color Dome; Diagnosys), rats are placed under isoflurane, pupils are drug-dilated, and electrodes are put on eyes (recording), cheeks (reference), and tail (ground). Eyes are flashed with light (10 cd.s/m²; 5 msec); and evoked retina photoreceptor potentials are recorded (a-waveform).

Bloodwork, Tissue Fatty Acids and Cytokines, and ¹⁹F-MRI Based Immune Cell Tracking:

Rat blood (cardiac puncture) is submitted to WRAIR Clinical Pathology for Complete Blood Count (CBC) and chemistry panel. Total fatty acid profiles of fresh liver are obtained, using lipid extraction and GC/MS (7890A/5975C; Agilent) methods. Cytokine levels of fresh brain are found, using protein lysates and immunoassay arrays (Luminex; R&D Systems). Macrophage immune cell deposits are revealed in eyes and brains of PFA-fixed rats, using a pre-injected (24 h) ¹⁹F-contrast agent (V-Sense; Celsense) and high resolution MRI scanning (Univ. of Pittsburgh).

Histopathology (H&E, Silver, Iba-1, and GFAP):

Following transcardial perfusion of rats with PFA, fixed eyes and brains are processed (FD Neurotechnologies) into sections and then H&E (eyes) and silver, Iba-1, and GFAP (brains) stained / immunohistochemistry (IHC) slides; which are examined by microscopy for immune cell infiltration and neurodegeneration.

Results

Diet Impact on Body Weights, Blood Glucose, and Liver Fatty Acids:

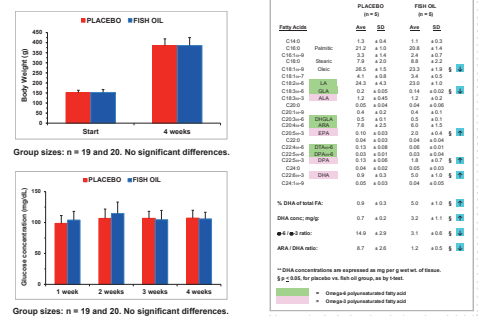
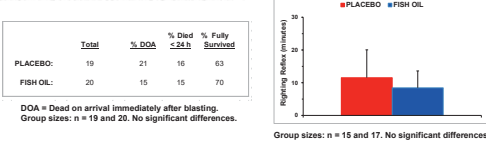


Figure 1. Body weights, blood glucose, and liver total fatty acids of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets for at least 4 weeks. Blood glucose was obtained by tail stick and a handheld diabetic monitor (Contour; Bayer); normal rats is 105 ± 13 mg/dL (n = 14).

Double Blast Impact on % Survival, Righting Reflex, and Terminal Bloodwork:



3 DAYS POST				7 DAYS POST			
PLACEBO (n=5)		FISH OIL (n=4)		PLACEBO (n=7)		FISH OIL (n=10)	
Test	Stat	Stat	Stat	Test	Stat	Stat	Stat
HCT (%)	39 ± 3	40 ± 1	38 ± 2	41 ± 1	39 ± 2	40 ± 1	38 ± 2
PLT (per µL)	1000 ± 100	1000 ± 100	1000 ± 100	1000 ± 100	1000 ± 100	1000 ± 100	1000 ± 100
WBC (per µL)	6000 ± 1000	7000 ± 2000	7000 ± 2000	7000 ± 2000	6000 ± 1000	6000 ± 1000	6000 ± 1000
NEUT (per µL)	500 ± 200	600 ± 200	600 ± 200	600 ± 200	500 ± 200	500 ± 200	500 ± 200
LYMPH (per µL)	1700 ± 1000	1000 ± 1000	1000 ± 1000	1000 ± 1000	1700 ± 1000	1000 ± 1000	1000 ± 1000
MONO (per µL)	100 ± 50	100 ± 50	100 ± 50	100 ± 50	100 ± 50	100 ± 50	100 ± 50
RBC (per µL)	4.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5
HAEMATOCRIT (%)	39 ± 3	40 ± 1	38 ± 2	41 ± 1	39 ± 2	40 ± 1	38 ± 2
TS (g/dL)	36 ± 2	36 ± 2	36 ± 2	36 ± 2	36 ± 2	36 ± 2	36 ± 2
ALT (U/L)	27 ± 10	27 ± 10	27 ± 10	27 ± 10	27 ± 10	27 ± 10	27 ± 10

Figure 2. Percent survival, righting reflex, and bloodwork of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at 3 and 7 days post-blast. WRAIR Clinical Pathology flagged any abnormal bloodwork values.

Double Blast Impact on Visual Acuties:

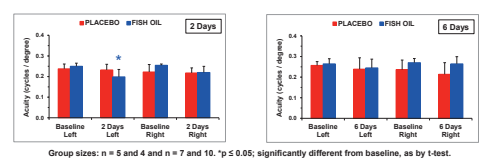


Figure 4. Visual acuity responses (cycles/degree) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 3 and 7 days post-blast.

Double Blast Impact on Electroretinography:

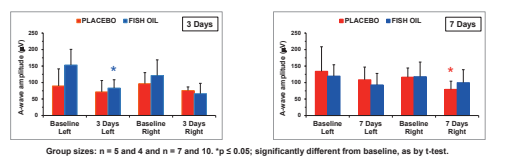


Figure 4. ERG amplitudes (µV) for a-wave signal responses (10 cd.s/m² flash) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, at baseline and 3 and 7 days post-blast.

Double Blast Impact on Cytokines and ¹⁹F-MRI:

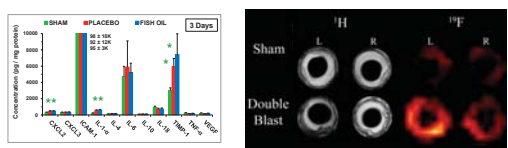


Figure 5. Brain (cerebellum) cytokine levels and ¹H- & ¹⁹F-MRI scans of eyes (left and right) from rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, at 3 days post-blast. MRI are from omega-3 sufficient rats (house chow).

Double Blast Impact on Histopathology:

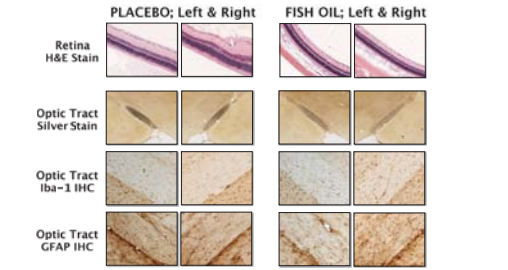


Figure 5. Histopathology of eyes (retina) and brains (optic tract) from two representative rats fed an omega-3 fatty acid deficient (placebo) or enriched (fish oil) diet, at 7 days post-blast. Magnification is 4 – 20x.

Summary and Conclusions

Our current findings in rats reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days), as with visual acuity and electroretinography testing. These deficits are accompanied by neurodegeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines, as obtained by immunoassay arrays, magnetic resonance imaging, and histopathology. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown slight ability to alleviate these acute injury events, despite having anti-inflammatory properties.

DISCLAIMER: Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. Opinions or assertions contained herein are private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals.

Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

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Blast injury is arguably the greatest threat to Warfighters in current campaigns, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain from blast shock waves. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast, which allows evaluations of neuronal injuries to the retina and brain. Our hypothesis is that immune cells play a primary role in exacerbating neurodegeneration following blast. Our objective is to monitor the nature and timing of neuroinflammation processes so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutrients known to be anti-inflammatory, e.g. omega-3 polyunsaturated fatty acids, on blast vulnerability. Adult male rats were fed for one month an omega-3 fatty acid deficient versus enriched (fish oil supplemented) diet. Blast injury was produced in anesthetized animals secured in a compressed air driven shock tube and then exposed twice (1 min interval) to blast over pressure waves (20 psi, 8 msec). At 3 to 28 days post-blast, retina and brain injuries were followed by electroretinography, visual acuity assessment, magnetic resonance imaging, histopathology, and cytokine level outcome measures. Our findings reveal that vision impairments occur early post-blast (i.e., within 7 days), and are accompanied by neurodegeneration in the retina and brain along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have so far shown slight, if any, ability to alleviate these acute injury events. Overall, our mission is to discover new drug treatments for blast-induced neurotrauma sustained by military personnel.

Supported by DoD grants from MOMRP and USAMRMC / CDMRP, #: W81XWH-14-2-0178.



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Background

Blast injury has emerged as arguably the greatest threat to Warfighters in current campaigns, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from shock waves. Despite the serious disability that vision loss represents, there are few animal studies that rigorously assessed blast wave injuries to the visual system. These studies often suffer from poor simulation of blast injuries or are limited in scope of outcome measures. None have studied the interplay of damage to the retina and brain. Thus, there is an urgent need to do advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role in dictating the extent of neuronal cell death in retina and brain following blast. Using adult rats exposed to shock tube generated blast waves, our objective is to longitudinally monitor up to 28 days the nature and timing of immune cell guided inflammatory processes in the injured retina and brain as to discern potential drug targets and therapeutic windows. We are also examining the impact of interventions known to be anti-inflammatory, i.e., giving the rats a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Overall, our mission is to provide data that will ultimately lead to discovery of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grant awards from the MOMP and USAMRMC / CDMRP/ Vision Research Program #: W81XWH-14-2-0178.

Introduction

□ Blast injuries have resulted since 2008 in more than 280,000 cases of traumatic brain injury in U.S. Soldiers, with frequent symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013).

□ While brain visual centers can be affected, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011).

□ Soldiers are issued protective goggles but still suffer eye injuries, such as from blast wave penetration (Weichel, 2008).

□ Few animal studies have tried to characterize visual system injuries as generated by rigorously simulated blast waves; and only three have evaluated drug treatments (review by DeMar, 2016).

□ Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010).

□ Metabolites of dietary omega-3 polyunsaturated fatty acids can suppress immune cell activation (Serhan, 2008, 2011, 2012).

References:

- Capo-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
- Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
- DeMar et al., 2016; Front. Neurol. 7: 59(1-12).
- Lemke et al., 2013; JAMA Ophthalmol. 131(12): 1602-1609.
- Serhan et al., 2008, 2010, 2011, & 2012; British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177(4): 1576-1591, Curr. Top. Med. Chem. 11(6): 629-647, & Chem. Rev. 111(10): 5922-5943.
- Weichel et al., 2008; Curr. Opin. Ophthalmol. 19(6): 519-525.

Aim of Study

In rats raised on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves, characterize up to 28 days out the visual dysfunction and underlying neuro-inflammation / degeneration due to retina and brain injuries, as by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Bloodwork, tissue fatty acids and cytokines, and Magnetic Resonance Imaging (MRI) based immune cell tracking.
- 3) Histopathology (H&E and silver stains; and GFAP & Iba-1 IHC).

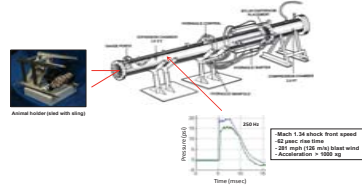
Materials and Methods

Animals and Dietary Manipulations:

Adult male Sprague Dawley rats (30 d-old) are fed for 4 weeks a chow devoid of long chain omega-3 polyunsaturated fatty acids (#: STLN; Purina Mills). Some animals are omega-3 supplemented with fish oil (ProOmega; Nordic Naturals), daily by gavage to provide 200 and 273 mg/kg of DHA and EPA. Placebo controls are given an equal volume of soybean oil.

Simulation of Primary Blast Wave Injuries:

□ Rats are exposed under isoflurane anesthesia to two blast over pressure waves, i.e., double blast (20 psi; 1min interval), in a right-side on position, using a compressed air driven shock tube.



Visual Acuity (Optokinetics):

Using an optokinetics device (Optomotry; Cerebral-Mechanics), rats are put on a pedestal in a chamber, where a rotating bar pattern is shown on four LCD monitors. Rotation speed is increased stepwise to narrow the bar's width; and visual acuity threshold (cycles/degree) is found, when animal ceases head-eye tracking movements (nystagmus). Separate eye acuities are determined by driving the rotation in opposite directions.

Electroretinography (ERG):

Rats are dark adapted for 16 h. Using a full field flash Ganzfeld ERG device (Color Dome; Diagnosys), rats are placed under isoflurane, pupils are drug-dilated, and electrodes are put on eyes (recording), cheeks (reference), and tail (ground). Eyes are flashed with light (10 cd.s/m²; 5 msec); and evoked retina photoreceptor potentials are recorded (a-waveform).

Bloodwork, Tissue Fatty Acids and Cytokines, and ¹⁹F-MRI Based Immune Cell Tracking:

Blood glucose is determined on living rats by tail stick and a handheld test-strip monitor (Contour; Bayer). Terminal rat blood (cardiac puncture) is submitted to WRAIR Clinical Pathology for Complete Blood Count (CBC) and chemistry panel. Total fatty acid profiles of fresh liver are obtained, by lipid extraction and GC/MS (7890A/5975C; Agilent) methods. Cytokine levels of fresh brain are found, using protein lysates and immunoassay arrays (rat 10-plex; Luminex, R&D Systems). Macrophage immune cell deposits are revealed in eyes and brains of PFA-fixed rats, using a pre-injected (24 h) ¹⁹F-contrast agent (V-Sense; Celsense) and high resolution MRI scanning (Univ. of Pittsburgh).

Histopathology (H&E, Silver, Iba-1, and GFAP):

Following transcardial perfusion of rats with PFA, fixed eyes and brains are processed (FD Neurotechnologies) into sections and then H&E (eyes), silver (brains), GFAP (both) and Iba-1(both) stained / immunohistochemistry (IHC) slides; which are examined by microscopy for signs of immune cell infiltration and neurodegeneration.

Results

Diet Impact on Body Weights, Blood Glucose, and Liver Fatty Acids:

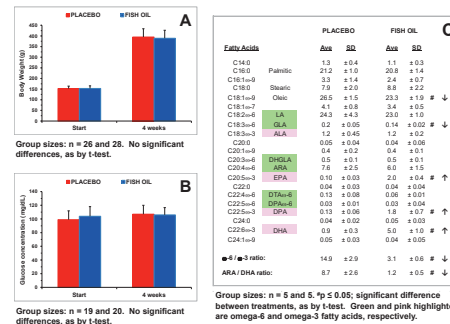


Figure 1. Body weights (A), blood glucose (B), and liver fatty acids (C) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets for 4 weeks.

Double Blast Impact on Survival, Righting Reflex, and Bloodwork:

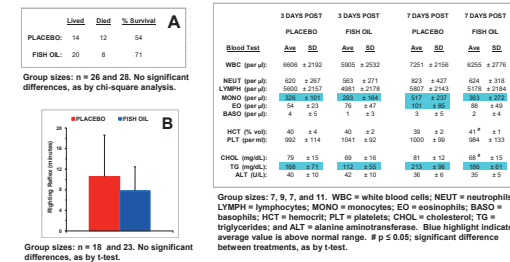


Figure 2. Percent survival (A), righting reflex (B), and bloodwork (C) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets immediately after blasting and at 3 and 7 days post-blast.

Double Blast Impact on Visual Acuities and Electroretinography (ERG) :

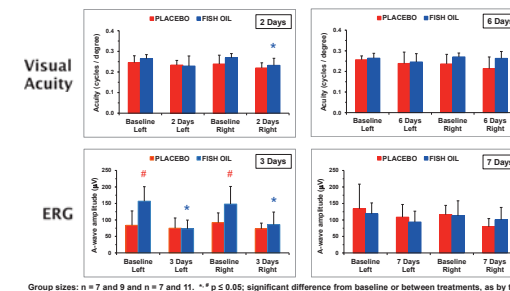
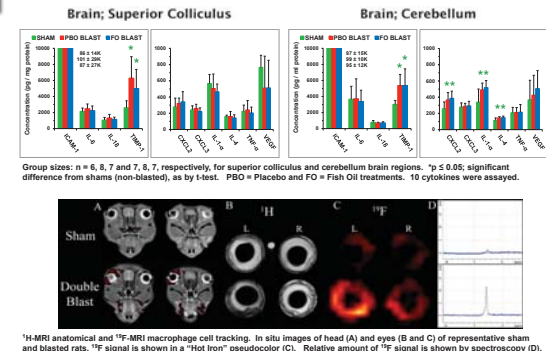


Figure 3. Visual acuities (cycles/degree) and ERG amplitudes (µV; a-wave) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 2 - 3 and 6 - 7 days post-blast.

Double Blast Impact on Cytokines and ¹⁹F-MRI:



Double Blast Impact on Histopathology:

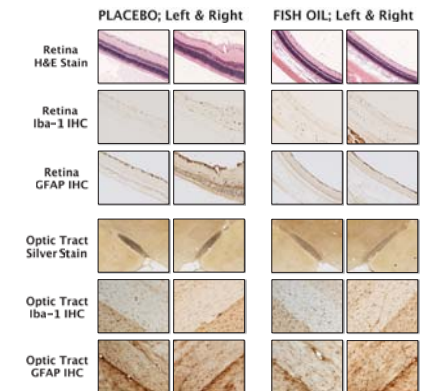


Figure 5. Histopathology of eyes (retina) and brains (optic tract) from two representative rats fed an omega-3 fatty acid deficient (placebo) or enriched (fish oil) diet, at 7 days post-blast. Magnification is 4 - 20x.

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